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ENZYMATIC PATHWAYS IN THE FORMATION OF SORBITOL AND GLYCEROL IN THE DIAPAUSING EGG OF THE SILKWORM, *BOMBYX MORI*—I ON THE POLYOL DEHYDROGENASES

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(*Received* 16 *October* 1959)

Abstract—In the previous papers (CHINO, 1957, 1958) it was reported that nearly all glycogen originally present in the silkworm egg was rapidly converted to sorbitol and glycerol during the diapause, and that glycogen was resynthesized from these polyols when diapause is brought to an end. The present study was undertaken to find the enzymatic pathways of sorbitol and glycerol formation, with special reference to the dehydrogenases of these polyols. The results obtained indicate that sorbitol is formed by the dehydrogenases catalysing the following reactions: glucose + TPNH $\rightleftarrows$ sorbitol + TPN^+ and glucose-6-phosphate or fructose-6-phosphate + TPNH $\rightleftarrows$ sorbitol-6-phosphate + TPN^+. Glycerol is formed by the enzymes catalysing the reactions: dihydroxyacetone phosphate + DPNH $\rightleftarrows$ α-glycerol phosphate + DPN^+ and glyceraldehyde or dihydroxy-acetone + TPNH $\rightleftarrows$ glycerol + TPN^+. These polyol dehydrogenases can be detected not only in the diapausing egg but also in the developing egg in which the accumulation of polyols never occurs.

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INTRODUCTION

IT was indicated in the previous papers (CHINO, 1957, 1958) that almost all glycogen initially present in the 'diapause' egg of the *Bombyx* silkworm was rapidly broken down with the onset of diapause and the end-product of this breakdown was not lactic acid but two polyols, sorbitol and glycerol. It was further shown that the resynthesis of glycogen from these polyols occurred concurrently with the termination of diapause. The present study has been undertaken to find the polyol dehydrogenases responsible for the formation of sorbitol and glycerol.

Almost all the dehydrogenases hitherto reported to be concerned in the formation of the polyols required DPN† or TPN as the coenzyme, and their reactions are as follows:

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† The following abbreviations will be used in this paper: DPN = diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; TPNH = reduced triphosphopyridine nucleotide; G-6-P = glucose-6-phosphate; F-6-P = fructose-6-phosphate; M-6-P = mannose-6-phosphate; FDP = fructose-1,6-diphosphate; GA = glyceraldehyde; DHA = dihydroxyacetone; GA-3-P = glyceraldehyde-3-phosphate; DHA-P = dihydroxyacetone phosphate; α-GP = α-glycerol phosphate.

Sorbitol formation

(1) fructose + DPNH⇌sorbitol + DPN^+
in mammalian liver and acetobacter (BLAKLEY, 1951; ARCUS and EDSON, 1956)

(2) glucose + TPNH⇌sorbitol + TPN^+
in mammalian seminal vesicle (HERS, 1956)

(3) G-6-P or F-6-P + TPNH⇌sorbitol-6-phosphate + TPN^+
in silkworm blood (FAULKNER, 1956b)

Glycerol formation

(4) DHA-P + DPNH⇌α-GP + DPN^+
in mammalian muscle (BARANOWSKI, 1949)

(5) GA or DHA + TPNH⇌glycerol + TPN^+
in silkworm blood (FAULKNER, 1958)

(6) DHA + DPNH⇌glycerol + DPN^+
in aerobacter (BURTON and KAPLAN, 1953)

The results to be described in this paper indicate that the silkworm egg contains the polyol dehydrogenases catalysing the reactions 2, 3, 4, and 5 listed above. These dehydrogenases are found not only in the diapausing eggs but also in developing eggs such as the pre-diapause and the 'non-diapause' eggs in which the accumulation of sorbitol and glycerol is never observed.

The work on these polyol dehydrogenases with some related experiments will be presented in this paper.

MATERIALS AND METHODS

Materials

Eggs and fifth instar larvae from the cross between Chinese and Japanese bivoltine races were used. Most experiments were carried out with the 'diapausing' egg from 45 to 60 days after oviposition, since the activities of the polyol dehydrogenases did not fluctuate within this age range. The methods for obtaining the so-called 'diapause' and 'non-diapause' eggs have been described in the previous paper (CHINO, 1957).

Preparation of enzyme solution

Enzyme solutions were prepared after FAULKNER's procedure (1958). One to 1·5 g of the eggs or larval tissues were ground with 5–10 ml of water at 0°C in a glass homogenizer. The homogenate was centrifuged at 14,500 *g* for 10 min at 0°C and the supernatant was transferred to a cellophane bag, and dialysed against 5 l. of distilled water with constant stirring for 18 hr at 1–3°C. After dialysis, the solution was filtered through Whatman No. 1 paper, and the filtrate was used as enzyme solution. The dialysed larval blood was also obtained by the method of FAULKNER (1956). Blood from the fifth instar larvae was diluted with an equal volume of chilled water, and dialysed as described above. The dialysed solution filtered through Whatman No. 1 paper was used as the enzyme solution.

Chemicals

DPN and TPN were 95 per cent pure, and DPNH and TPNH were 90 per cent pure. These co-enzymes were purchased from Sigma Chemical Co., St. Louis, Missouri; G-6-P, F-6-P, M-6-P, FDP, DHA, DL-GA, and crystalline aldolase from Nutritional Biochemicals Co., Cleveland, Ohio; α-GP from Fluka Co., Switzerland. F-6-P and FDP were purified by several re-precipitations of the barium salts from water–ethanol mixtures.

Sorbitol-6-phosphate and mannitol-6-phosphate were prepared by reduction of the sodium salts of G-6-P and M-6-P respectively with a sodium borohydride solution by the procedure of ABDEL-AKHER *et al.* (1951). Periodate oxidation and chromotropic acid treatment of the product gave a formaldehyde : phosphorus ratio of 1·08 : 1 for sorbitol-6-phosphate and 0·96 : 1 for mannitol-6-phosphate respectively, the expected ratio for these two polyol phosphates being 1 : 1. D- and L-GA were prepared by the method of PERLIN and BRICE (1956). The mixture of DHA-P and GA-3-P (triose phosphate mixture) was prepared by incubating FDP with the crystalline aldolase.

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Spectrophotometric determination of polyol dehydrogenases

The polyol dehydrogenase activities were assayed by reading optical density at 340 mμ with silica cells (1 cm light path) in a Hitachi spectrophotometer (model EPU-2). One unit of enzyme was defined as the amount causing an increase or a decrease of optical density by 0·01/min in the standard test system which contained the following in a final volume of 3 ml: tris-hydroxymethyl-aminomethane (tris) buffer (20 mM) or glycine buffer (20 mM); $MgSO_4$ (4 mM); DPN, TPN, DPNH, or TPNH (0·07 mM); substrate; 0·5–1 ml enzyme solution.

Protein was determined by the method of LOWRY *et al.* (1951).

RESULTS

1. *TPN-linked polyol dehydrogenases*

Reduction of sugars and sugar phosphates. Recently FAULKNER (1956a, b) has reported that larval blood of the silkworm contains 'malic enzyme', and that the oxidation of L-malate can be coupled with the reduction of G-6-P or GA. As shown in Fig. 1, a very high activity of malic enzyme was also observed in the dialysed egg extract. TPN added initially was completely reduced by incubation with egg extract containing L-malate. Addition of DL-GA caused the re-oxidation of TPNH, whereas addition of G-6-P failed to re-oxidize TPNH. It appears that polyol dehydrogenases in the egg can reduce GA but not G-6-P in the presence of TPNH. The evidence presented by FAULKNER (1958), however, shows that the reduction of GA and G-6-P and/or other sugar phosphates with TPNH is catalysed by the same enzyme. The discrepancy observed between the egg extract and the larval blood can be explained by the presence of G-6-P dehydrogenase in the former and the absence of it in the latter. If the dialysed

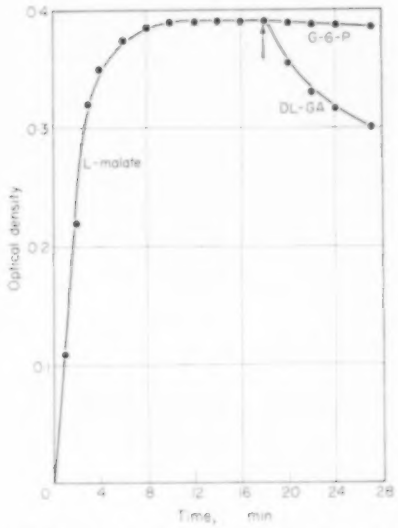

FIG. 1. Coupling of oxidation of L-malate and reduction of G-6-P or DL-GA. Initial incubation mixture: 20 mM tris buffer pH 7·5, 4 mM $MgSO_4$, 3 mM L-malate, 0·06 mM TPN, 0·5 ml dialysed egg extract from the diapausing egg. At 18 min, 10 μmoles DL-GA or G-6-P was added. Final volume 3·3 ml. Incubation at 22°C. Optical density readings have been corrected for the dilution after addition of DL-GA or G-6-P. Each point has been corrected for the readings in the absence of substrate.

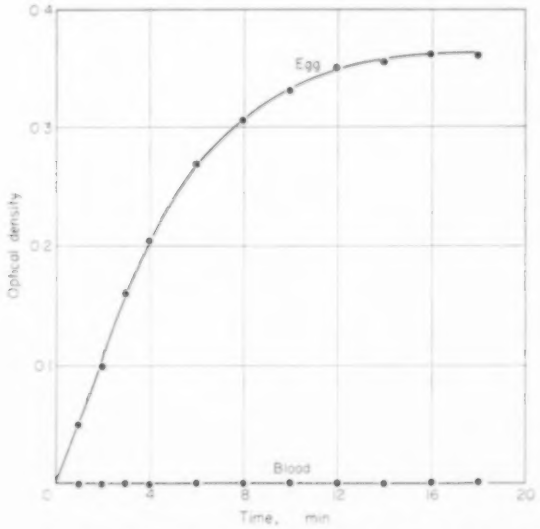

FIG. 2. Glucose-6-phosphate dehydrogenase in the egg and the larval blood. Incubation mixture: 20 mM tris buffer pH 7·5, 4 mM MgSO, 3 mM G-6-P, 0·06 mM TPN, 0·5 ml dialysed extract from the diapausing egg or 0·5 ml dialysed larval blood. Final volume 3 ml. Incubation at 22°C. Each point has been corrected for the readings in the absence of substrate.

egg extract has contained G-6-P dehydrogenase, re-oxidation of TPNH with G-6-P should not have been detected as the result of the following reactions:

(1) G-6-P + TPNH ⇌ sorbitol-6-phosphate + TPN^+;

(2) G-6-P + TPN^+ ⇌ 6-phosphogluconate + TPNH.

TABLE 1—RATES OF REDUCTION OF SUGARS OR SUGAR PHOSPHATES WITH TPNH AND THOSE OF OXIDATION OF POLYOLS OR POLYOL PHOSPHATES WITH TPN

The dialysed extract from the diapausing egg was tested under the standard conditions described in 'Methods'. The activities are expressed as the rate at which the test substrate was reduced or oxidized compared with that for DL-GA, which was arbitrarily fixed at 100. Incubation at 23°C

Substrate	Final conc. (mM)	Coenzyme	pH	Relative activity
DL-Glyceraldehyde	4	TPNH	7·5	100
D-Glyceraldehyde	4	TPNH	7·5	100
L-Glyceraldehyde	4	TPNH	7·5	63
Dihydroxyacetone	4	TPNH	7·5	9
Dihydroxyacetone	10	TPNH	7·5	25
Glucose-6-phosphate	4	TPNH	7·5	Not demonstrable
Fructose-6-phosphate	4	TPNH	7·5	10
Glucose	4	TPNH	7·5	Not demonstrable
Glucose	50	TPNH	7·5	59
Fructose	50	TPNH	7·5	0
Sorbose	50	TPNH	7·5	0
Triose phosphate mixture	4	TPNH	7·5	0
Sorbitol	10	TPN	8·5	Not demonstrable
Sorbitol	50	TPN	8·5	37
Sorbitol-6-phosphate	4	TPN	8·5	19*
Sorbitol-6-phosphate	10	TPN	8·5	34*
Mannitol	100	TPN	8·5	0
Mannitol-6-phosphate	10	TPN	8·5	0
Glycerol	100	TPN	8·5	0
Glycerol	100	TPN	9·5	0
α-Glycerol phosphate	10	TPN	8·5	0

* The values do not represent the true rate of sorbitol-6-phosphate oxidation, because G-6-P formed by the oxidation should be further oxidized to 6-phosphogluconate by G-6-P dehydrogenase, resulting in an additional increase of TPNH.

In fact, as shown in Fig. 2, a very high activity of G-6-P dehydrogenase was observed in the egg extract, whereas practically no activity was detected in the larval blood. Furthermore, from the later experiment in which oxidation of sorbitol-6-phosphate with TPN can be demonstrated, it is evident that the dialysed egg extract contains the enzyme catalysing reaction 1.

According to FAULKNER (1958), the TPN-linked polyol dehydrogenase in the larval blood can rapidly reduce sugars with two to four carbons and slowly reduce pentoses, whereas it fails to reduce unsubstituted hexoses. The data presented in Fig. 3 and Table 1 indicate that in the dialysed egg extract glucose of high concentration (50 mM) can be reduced to a considerable extent with TPNH,

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whereas hardly any reduction occurs when the concentration of the sugar is low (4 mM). It seems desirable, therefore, to determine whether or not the reductions of GA and glucose are catalysed by the same enzyme. To this end, the reduction rates of GA and glucose respectively and in mixture were compared. If two enzymes were involved, the reduction rate of the mixture should approach the sum of the rates obtained with the substrate present separately. In a typical experiment, the rate of reduction with DL-GA (4 mM) alone was 0·0059 optical density/min mg protein and that with glucose (50 mM) was 0·0032/min mg protein, whereas their mixture was reduced at a rate of 0·0088/min mg protein, which is nearly the sum of the individual rates.

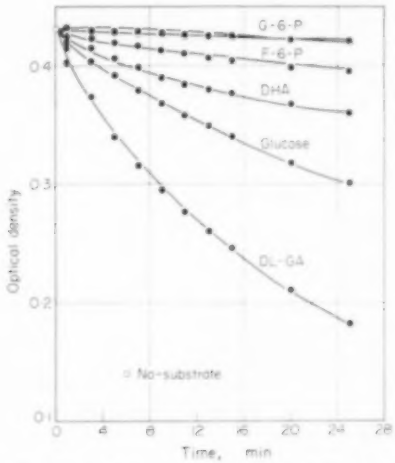

FIG. 3. Reduction of glucose, G-6-P, F-6-P, DHA, and DL-GA with TPNH. Incubation mixture: 20 mM tris buffer pH 7·5, 4 mM $MgSO_4$, 0·07 mM TPNH, substrate (50 mM glucose, 3 mM G-6-P, 3 mM F-6-P, 10 mM DHA, or 3 mM DL-GA), 0·5 ml dialysed extract from the diapausing egg. Final volume 3 ml. Incubation at 23°C.

It is also apparent from Fig. 3 and Table 1 that the dialysed egg extract, as well as the larval blood in FAULKNER's report, can reduce F-6-P and DHA in addition to glucose and GA in the presence of TPNH.

Oxidation of polyols and polyol phosphates (reversibility). Although the equilibrium of the reactions catalysed by the DPN- or TPN-linked polyol dehydrogenases is known to favour the reduction of sugar, the reverse reactions could be demonstrated by addition of some polyols or their phosphates of high concentration at relatively high pH values.

Fig. 4 and Table 1 show that sorbitol and sorbitol-6-phosphate are oxidized to a considerable extent with TPN. By these results it is further shown that the egg extract contains the dehydrogenases catalysing the reactions: glucose + TPNH = sorbitol + TPN^+ and G-6-P + TPNH = sorbitol-6-phosphate + TPN^+. These TPN-linked polyol dehydrogenases were also distributed in the

various larval tissues such as blood, mid-gut, and fat body (Table 2). Therefore, if FAULKNER (1958) had used glucose of a higher concentration, he would have observed the reduction of glucose with TPNH.

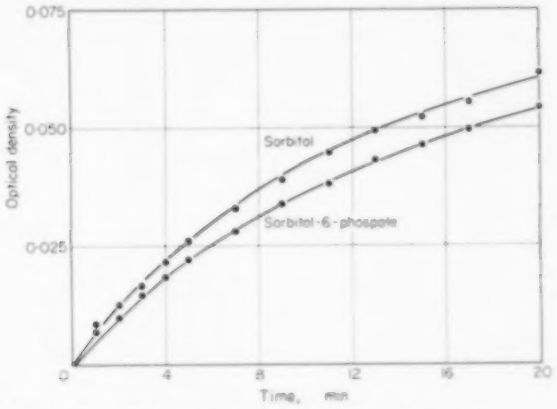

FIG. 4. Oxidation of sorbitol and sorbitol-6-phosphate with TPN. Incubation mixture: 20 mM tris buffer pH 8·5, 4 mM $MgSO_4$, 0·07 mM TPN, 50 mM sorbitol, or 10 mM sorbitol-6-phosphate, 0·5 ml dialysed extract from the diapausing egg. Final volume 3 ml. Incubation at 23°C. Each point has been corrected for the readings in the absence of substrate.

As shown in Table 1, the oxidation of glycerol (100 mM) with TPN could not be demonstrated. This can be taken to indicate that the equilibrium of the reaction, GA or DHA + TPNH⇌glycerol + TPN^+, very much favours the reduction of the sugars, as was indicated in FAULKNER's report (1958). Table 1 further

TABLE 2—DISTRIBUTION OF THE TPN-LINKED POLYOL DEHYDROGENASES IN THE VARIOUS TISSUES OF THE SILKWORM

The standard test system was used. Incubation at 23°C at pH 8·5

Substrate	Final conc. (mM)	Coenzyme	Tissue	Units/mg of protein
Sorbitol	50	TPN	Diapausing egg	0·16
			Larval blood	0·10
			Larval mid-gut	0·11
			Larval fat-body	0·09
Sorbitol-6-phosphate	10	TPN	Diapausing egg	0·18*
			Larval blood	0·12

* See the note of Table 1.

shows that in the presence of TPN the dialysed egg extract cannot oxidize mannitol-6-phosphate. Accordingly, one can assume that the only likely product of the reduction of F-6-P with TPNH is sorbitol-6-phosphate, although the reduction product of F-6-P would be either sorbitol-6 or mannitol-6-phosphate.

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2. *DPN-linked polyol dehydrogenases*

α-Glycerol phosphate dehydrogenase. ZEBE and MCSHAN (1957) and CHEFURKA (1958) have reported that various insect tissues contain α-GP dehydrogenase with very high activity. The presence of this enzyme with a high activity was also observed in the dialysed egg extract. When triose phosphate mixture (DHA-P and GA-3-P) and DPNH were incubated with the egg extract, a rapid oxidation

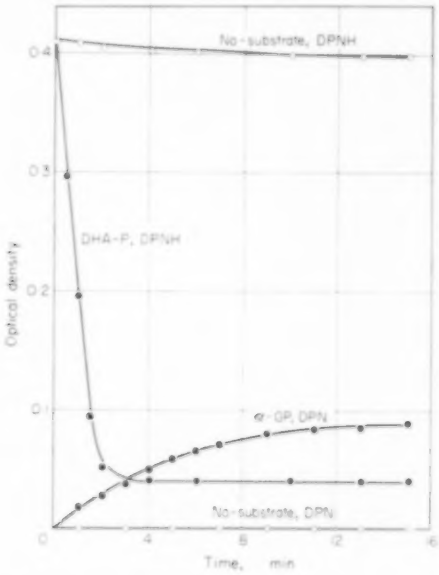

FIG. 5. Reduction of DHA-P with DPNH and oxidation of α-GP with DPN. Incubation mixture: 20 mM tris buffer pH 8·5, 4 mM $MgSO_4$, 0·07 mM DPNH or DPN, 3 mM triose phosphate (DHA-P and GA-3-P) or 10 mM α-GP, 0·5 ml dialysed extract from the diapausing egg. Final volume 3 ml. Incubation at 22°C.

of DPNH was observed (Fig. 5). Fig. 5 shows further that the reverse reaction can be demonstrated by incubating α-GP (10 mM) and DPN with the enzyme. Both the reduction of DHA-P and the oxidation of α-GP had their optimal pH at around 8·5 (Table 3).

As indicated in Table 3, the reduction of DL-GA, DHA, glucose, fructose, sorbose, and G-6-P with DPNH as well as the oxidation of glycerol, sorbitol, mannitol, sorbitol-6, and mannitol-6-phosphate with DPN was never observed in the dialysed egg extract. However, when F-6-P and DPNH were incubated with the dialysed egg extract, a rapid oxidation of DPNH occurred after an appreciable time-lag (Fig. 6). The presence of a lag, together with the fact that sorbitol-6 and mannitol 6-phosphate cannot be oxidized with DPN, suggests that the oxidation of DPNH does not represent the direct reduction of F-6-P

with DPNH. The author assumes that the time-lag represents the intermediations of the following reactions:

(1) F-6-P $\overset{\text{aldolase}}{\rightleftharpoons}$ GA-3-P + DHA.

(2) GA-3-P $\overset{\text{triose ph. isomerase}}{\rightleftharpoons}$ DHA-P.

(3) DHA-P + DPNH $\overset{\alpha\text{-GP dehydrogenase}}{\rightleftharpoons}$ α-GP + DPN^+.

In fact, an addition of $CuSO_4$ (0·2 mM), which is known as a strong inhibitor of aldolase, greatly prolonged the lag period (Fig. 6). Therefore, it is apparent

TABLE 3—RATES OF REDUCTION OF SUGARS AND SUGAR PHOSPHATES WITH DPNH AND THE RATES OF OXIDATION OF POLYOLS AND POLYOL PHOSPHATES WITH DPN

The acitivities are expressed as the rate at which the test substrate was reduced or oxidized compared with the reduction rate of DL-GA with TPNH, which was arbitrarily fixed at 100. Other explanations are the same as those in Table 1.

Substrate	Final conc. (mM)	Coenzyme	pH	Relative activity
Triose phosphate mixture	4	DPHN	7·5	500
Triose phosphate mixture	4	DPNH	8·5	1310
Triose phosphate mixture	4	DPNH	9·0	730
DL-Glyceraldehyde	4	DPNH	7·5	0
Dihydroxyacetone	10	DPHN	7·5	0
Glucose-6-phosphate	4	DPNH	7·5	0
Glucose	50	DPNH	7·5	0
Fructose	50	DPNH	7·5	0
Sorbose	50	DPNH	7·5	0
α-Glycerol phosphate	4	DPN	8·5	50
α-Glycerol phosphate	10	DPN	7·5	14
α-Glycerol phosphate	10	DPN	8·0	36
α-Glycerol phosphate	10	DPN	8·5	68
α-Glycerol phosphate	10	DPN	9·0	55
Sorbitol	50	DPN	8·5	0
Sorbitol	50	DPN	9·5	0
Sorbitol-6-phosphate	10	DPN	8·5	0
Mannitol	50	DPN	8·5	0
Mannitol	50	DPN	9·5	0
Mannitol-6-phosphate	10	DPN	8·5	0
Glycerol	100	DPN	8·5	0
Glycerol	100	DPN	9·5	0

that aldolase in the silkworm egg can split F-6-P in addition to the usual substrate, FDP, as was reported by DOUNCE *et al.* (1950) on the mammalian muscle. This splitting produces GA-3-P and DHA, and the former should be reduced

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to α-GP with DPNH and the latter may be reduced to glycerol with TPNH in the presence of the TPN-linked polyol dehydrogenase described in the previous section.

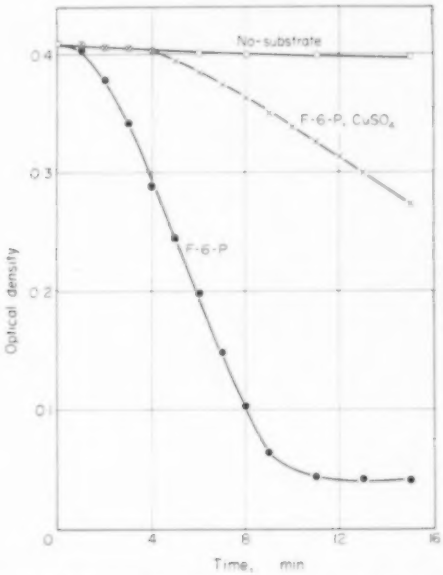

FIG. 6. Reduction of F-6-P with DPNH and the effect of cupric ion. Incubation mixture: 20 mM tris buffer pH 8·5, 4 mM $MgSO_4$, 0·07 mM DPNH, 3 mM F-6-P, 0·5 ml dialysed extract from the diapausing egg. 0·3 ml of 2 mM $CuSO_4$ was added. Final volume 3 ml. Incubation at 22°C.

3. *Distribution of polyol dehydrogenases in the 'diapause' and the 'non-diapause' eggs*

From the results described above, it is concluded that the diapausing egg contains the polyol dehydrogenases catalysing the following reactions: (1) glucose + TPNH ⇌ sorbitol + TPN^+; (2) G-6-P or F-6-P + TPNH ⇌ sorbitol-6-phosphate + TPN^+; (3) GA or DHA + TPNH ⇌ glycerol + TPN^+; and (4) DHA-P + DPNH ⇌ α-GP + DPN^+. Since reactions 2 and 3 are catalysed by the same enzyme, as already noted, it can be concluded that the diapausing egg contains three kinds of polyol dehydrogenases responsible for the formation of sorbitol and glycerol, namely, TPN-linked sorbitol dehydrogenase, TPN-linked glycerol or sorbitol-6-phosphate dehydrogenase, and DPN-linked α-GP dehydrogenase.

The activities of these three polyol dehydrogenases were examined during various stages of 'diapause' and 'non-diapause' eggs. The results are shown in Table 4. All these enzymes were found not only in the diapausing egg but also in the developing egg. It should be noted that the activities are not necessarily high even in stages in which the formation of sorbitol and glycerol takes place rapidly.

TABLE 4—DISTRIBUTIONS OF POLYOL DEHYDROGENASES IN 'DIAPAUSE' AND 'NON-DIAPAUSE' EGGS AND THE CHANGE IN ENZYME ACTIVITIES WITH EMBRYONIC STAGE

The test system for TPN-linked glycerol dehydrogenase contained: DL-GA (4 mM), TPNH (0·07 mM), at pH 7·5; that for TPN-linked sorbitol dehydrogenase contained: sorbitol (100 mM), TPN (0·1 mM), at pH 8·5; that for α-GP dehydrogenase contained: α-GP (10 mM), DPN (0·1 mM), at pH 8·5. Other conditions were the same as those of standard system. Incubation at 25°C.

Kind of eggs	Stage of eggs	Polyol dehydrogenase activity (units/mg of protein)		
		TPN-linked glycerol dehydrogenase	TPN-linked sorbitol dehydrogenase	α-Glycerol phosphate dehydrogenase
Diapause egg	1 day after oviposition (pre-diapause stage)	0·57	0·15	0·05
	3 days after oviposition (glycogen rapidly decreasing phase)	0·21	0·09	0·15
	6 days after oviposition (glycogen rapidly decreasing phase)	0·14	0·08	0·27
	11 days after oviposition (glycogen slowly decreasing phase)	0·15	0·08	0·35
	20 days after oviposition (glycogen slowly decreasing phase)	0·24	0·09	0·40
	45 to 65 days after oviposition (glycogen minimum stage)	0·54	0·21	0·40
	3 days in post-diapause at 25°C (glycogen recovering phase)	0·34	0·25	0·63
Non-diapause egg	1 day after oviposition	0·68	0·18	0·05
	4·5 days after oviposition (blastokinesis)	0·55	0·17	0·23
	8·5 days after oviposition (1 day before hatching)	0·31	0·12	0·41

4. *Comparison of α-glycerol phosphate dehydrogenase activity and lactic dehydrogenase activity*

According to ZEBE *et al.* (1957) and CHEFURKA (1958), some insect tissues, especially thoracic muscles, contain an α-GP dehydrogenase with very high activity, whereas they contain lactic dehydrogenase only of very weak activity.

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These observations seem to suggest glycerol accumulation in the diapausing egg.

An assay of lactic dehydrogenase activity of the diapausing egg was undertaken by incubating pyruvate and DPNH with the egg extract. The results shown in Table 5 indicate that activity of lactic dehydrogenase is much lower than that of α-GP dehydrogenase, the ratio of two activities being 1 : 20.

TABLE 5—α-GLYCEROL PHOSPHATE DEHYDROGENASE AND LACTIC DEHYDROGENASE IN THE DIAPAUSING EGG

Incubation mixture for α-GP dehydrogenase contained: 4 mM triose phosphate mixture (DHA-P and GA-3-P), 0·07 mM DPNH, 20 mM tris buffer at pH 8·5; that for lactic dehydrogenase contained: 4 mM pyruvate, 0·07 mM DPNH, tris buffer at pH 7·5. Other conditions were the same as those of the standard test condition. Incubation at 23°C.

Enzyme	Activity (units/mg of protein)	Ratio of activity
α-GP dehydrogenase	7·07	20·2
Lactic dehydrogenase	0·35	1

DISCUSSION

From the results described above, it is apparent that the silkworm egg contains the polyol dehydrogenases catalysing the following reactions: (1) glucose + TPNH ⇌ sorbitol + TPN^+; (2) G-6-P or F-6-P + TPNH ⇌ sorbitol-6-phosphate + TPN^+; (3) GA or DHA + TPNH ⇌ glycerol + TPN^+; and (4) DHA-P + DPNH ⇌ α-GP + DPN^+. Of these four reactions, three require TPN as the co-enzyme. Therefore, when sorbitol and glycerol are formed in the diapausing egg, a large amount of TPNH should be needed. In this connexion, it should be recalled that a considerable amount of TPNH is generated from the pentose phosphate cycle, the overall reaction of which can be given as follows:

(1) G-6-P + 6 TPN^+ ⇌ 3 CO_2 + GA-3-P + 6 TPNH.

The presence of this enzyme system in some insect tissues has been shown by CHEFURKA (1958). The present experiment has also revealed that the silkworm egg contains G-6-P dehydrogenase, which is an important member of the cycle.

Now let us consider how much glycogen can be oxidized via the pentose phosphate cycle when the eggs enter a state of diapause. According to the data presented in the previous paper (CHINO, 1958), the amount of glycogen utilized and that of sorbitol and glycerol formed during the period from 1 day (the onset of diapause) to 50 days (the glycogen minimum stage) after oviposition are as follows:

glycogen utilized: 194 μmoles as glucose equivalent/g of egg;
sorbitol formed: 121 μmoles/g of egg;
glycerol formed: 110 μmoles/g of egg.

From the above values, it is evident that all of glycogen utilized is not converted to sorbitol and glycerol. If one assumes that the difference can be attributed to the oxidation of glycogen via the pentose phosphate cycle, and if this amount is taken as x μmoles (as glucose equivalent), the amount of glycogen which can be converted to glycerol via the Embden–Meyerhof pathway is considered to be $194-(121+x)$ μmoles. At the same time, x μmoles of GA-3-P should be generated from the pentose phosphate cycle (see equation 1), all of which can be further converted to glycerol. Accordingly, the following equation may be established:

$$2\times\{194-(121+x)\}+x=110,$$
$$\text{therefore} \qquad x=36.$$

If 36 μmoles of glycogen (as glucose equivalent) is oxidized via the pentose phosphate cycle, 216 μmoles of TPNH should be generated (see equation 1). As already indicated, all the polyol dehydrogenases responsible for sorbitol formation require TPN alone as the co-enzyme. Therefore, when 121 μmoles of sorbitol is formed, the equivalent amount of TPNH is necessary. On the other hand, the polyol dehydrogenases in glycerol formation require TPN or DPN. However, since the activity of α-GP dehydrogenase is very high in the egg, it is likely that a part of the glycerol can be formed by the action of this enzyme, thus it is probable that the requirement of TPNH for glycerol formation may be not so high.

From the above considerations, it may be concluded that a large amount of TPNH required for polyol formation during diapause is generated from the pentose phosphate cycle, even if the difference between the amount of glycogen utilized and the sum of sorbitol and glycerol formed is not necessarily due to the oxidation of glycogen via the cycle. As another possibility, one can assume that a small amount of TPNH necessary for polyol formation is also generated from oxidation of malate by malic enzyme found in the egg. These possibilities are supported by the evidence reported previously (CHINO, 1958), indicating that the respiratory quotient becomes considerably greater than unity as soon as polyol formation begins at the onset of diapause. In this connexion, it may be valuable to recall an interesting observation of KAPLAN *et al.* (1956) that in rat liver mitochondria the electron transport from TPNH to oxygen is slow and no oxidative phosphorylation occurs in TPNH oxidation via the electron transport system. From these results, they have suggested that the prime function of TPNH is to serve as a reducing agent for a synthetic reaction involving a reduction in some physiological conditions.

Recently KUBISTA (1958) and CHEFURKA (1958) have found that in some insect tissues, especially in the thoracic muscle, the end product of glycolysis is not lactic acid but mainly α-GP and pyruvic acid. Chefurka has further shown that in these tissues the level of lactic dehydrogenase is much lower than that of α-GP dehydrogenase, thus resulting in the formation of α-GP instead of lactic acid. The present study has also revealed a similar situation in the diapausing egg of the silkworm. The large difference in the activities of the two enzymes may be one of the factors causing the accumulation of glycerol, but it is not considered

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to be a major factor for the following reason: in the thoracic muscle α-GP is formed by the dismutation of triose phosphate, whereas in the diapausing egg of the silkworm the oxidation of triose phosphate can scarcely proceed because glycogen is nearly converted to sorbitol and glycerol.

Furthermore, it should be pointed out that all polyol dehydrogenases responsible for sorbitol and glycerol formation are distributed equally in the diapausing egg, in the pre-diapausing egg, as well as in the 'non-diapause' egg; in the latter two accumulation of polyols has never occurred. This fact suggests that in developing eggs the reactions of the polyol dehydrogenases favour the oxidation of polyols, whereas in diapausing eggs these reactions favour sugar reduction, namely, polyol formation. Accordingly, it can be assumed that a primary factor causing the accumulation of sorbitol and glycerol only during diapause is a physicochemical change evoked at the onset of diapause, which should promote the reduction of sugar. Probably such a physicochemical change would be accompanied by blocking of the electron transport from TPNH and DPNH to oxygen which results in the accumulation of TPNH and DPNH, and, in turn, favours the reduction of sugar.

SUMMARY

In order to find the enzymatic pathways of sorbitol and glycerol formation in the diapausing egg of the silkworm, studies on polyol dehydrogenases, together with some related experiments, were carried out.

(1) The silkworm egg contained polyol dehydrogenases catalysing the following reactions:

$$\text{glucose} + \text{TPNH} \rightleftharpoons \text{sorbitol} + \text{TPN}^+;$$

$$\text{G-6-P or F-6-P} + \text{TPNH} \rightleftharpoons \text{sorbitol-6-phosphate} + \text{TPN}^+;$$

$$\text{GA or DHA} + \text{TPNH} \rightleftharpoons \text{glycerol} + \text{TPN}^+;$$

$$\text{DHA-P} + \text{DPNH} \rightleftharpoons \alpha\text{-GP} + \text{DPN}^+.$$

(2) All these polyol dehydrogenases were found not only in the diapausing egg but also in the developing egg in which the accumulation of sorbitol and glycerol is never seen. Therefore, it was assumed that the primary factor causing the accumulation of these polyols only during diapause is some physicochemical change which arises at the onset of diapause. Such a change should be such as to bring about a block of the electron transport system of the egg, which results in the accumulation of TPNH and DPNH, favouring the formation of the polyols.

(3) From the stoichiometric evidence, it was suggested that TPNH necessary for sorbitol and glycerol formation in the diapausing egg should be generated mostly from the pentose phosphate cycle.

(4) In the diapausing egg, the activity of lactic dehydrogenase was much lower than that of α-glycerol phosphate dehydrogenase. This may be one of the factors for the accumulation of glycerol during diapause.

(5) Aldolase in the silkworm egg could split fructose-6-phosphate in addition to its usual substrate, fructose-1,6-diphosphate, producing the mixture of dihydroxyacetone and glyceraldehyde-3-phosphate.

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THE ROLE OF OLFACTION IN THE STIMULATION OF OVIPOSITION IN THE BLOWFLY, *PHORMIA REGINA**

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Abstract—The odour of a liquid medium containing powdered milk and yeast stimulates *Phormia regina* to oviposit. In the presence of a sufficiently high odour concentration, contact stimulation by the same medium does not increase egg laying. The receptors chiefly involved in the reception of the odour are located on the antennae and/or palps. Evidence is given for olfactory reception by the ovipositor.

INTRODUCTION

THE preferred oviposition sites of a large number of insects are known but very little information exists about the sensory processes involved in the selection of these sites. The ability of some mosquitoes to discriminate between different salt solutions as sites for oviposition has been shown to depend on the stimulation of contact chemoreceptors on the tarsi (WALLIS, 1954; HUDSON, 1956). DETHIER (1957) has brought together the available information about host recognition in a number of insect parasites.

The oviposition behaviour of insects is usually complex (e.g. MAKINGS, 1958; CRAGG, 1956) but can generally be divided into two major phases: that involving attraction to the general area of the oviposition site and that involving the acts leading up to oviposition itself which occur after the area has been reached. The experiments on *Phormia regina* described in this paper deal only with the latter phases and were designed not only to determine the role of olfactory stimuli in inciting flies to oviposit but also to locate the receptors concerned. The study was restricted almost entirely to an examination of the role of olfaction because it was found that, at least in the presence of a sufficient odour concentration, contact stimuli play little or no part. This means that a critical study of the role of contact chemical stimuli can only be carried out using a non-volatile oviposition stimulant. At the present time no such substance is known.

METHODS

A. *Treatment of flies*

About 100 flies were allowed to emerge into a wire gauze cage measuring 10 × 10 × 10 in. On the second and third days the flies were provided with liver

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in addition to the sucrose and water which were always present. By the sixth day most of the female flies were ready to oviposit and at this age were ready for use.

B. *The oviposition medium*

This consisted of 10 per cent Kilm powdered milk and 10 per cent dry brewer's yeast (Type 2019, Standard Brands) in water.

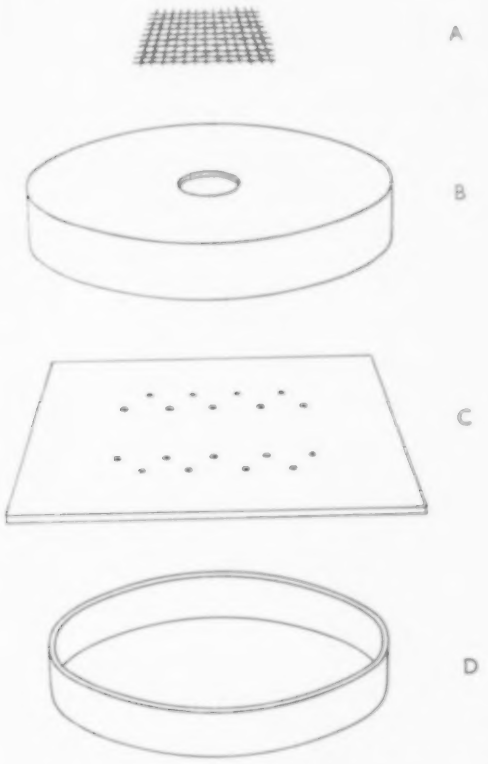

FIG. 1. Exploded view of plastic oviposition container. A. Gauze. B. Inverted petri dish. C. Plastic plate. D. Lower petri dish.

C. *Oviposition sites*

Fig. 1 shows the plastic oviposition container in which the flies were confined. This is based on that used by A. J. NICHOLSON (personal communication) and was made up of three main parts. A 4 in. square of clear $\frac{1}{8}$ in. acrylic plastic, in which there were eighteen holes of diameter 0·07 in., was placed over a 4 in. petri dish. A second petri dish, in the centre of which was a hole $\frac{1}{2}$ in. dia., was inverted on top of the plate. The hole was covered with wire gauze and six female flies were confined under the inverted dish. In this apparatus it was possible to provide

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liquid medium either below or on the plate or both. The medium below the plate was out of reach of the ovipositors of the flies and hence provided only an olfactory stimulus.

The flies laid nearly all their egg masses through the holes provided in the plastic plate. BARTON BROWNE (1958) has shown that females of *Lucilia cuprina* are stimulated to lay by the presence of already ovipositing females and this is also known to be so in *P. regina* (BARTON BROWNE, unpublished). The use of separate holes in the above types of apparatus ensures that groups of ovipositing females cannot occur and therefore minimizes the chance of interference from this complicating factor.

A drop of 0·1 M sucrose was provided on the plates in experiments designed to study the effect of the presence of the odour of the medium on the oviposition of either intact females or females deprived of organs suspected of bearing relevant sensilla. The presence of this solution was found to increase the number of egg masses laid and to reduce the variance. Because of its stimulating effect the drop of solution was omitted from preparations in experiments in which the oviposition in the presence of the odour of the medium of intact females was compared with that of females from which organs had been removed.

D. *Timing and duration of experiments*

In all experiments the flies were placed in the oviposition containers at the end of the afternoon and were allowed to remain overnight. Most experiments were allowed to continue until the number of egg masses laid by the series of flies under the more favourable of the conditions provided was equal to about half the number of flies present. If it was found necessary to allow an experiment to continue for a long period the preparations were cleared of eggs at intervals so that none hatched. The medium was renewed every morning and evening.

RESULTS

A. *The relative importance of olfactory and gustatory stimuli*

1. Six intact female flies were placed in each of twenty-four plastic oviposition containers. In twelve of these there was medium both beneath and on the plastic plate, whereas in the other twelve there was medium beneath only. In both series a drop of 0·1 M sucrose solution was provided on the plate. The mean number of egg masses per preparation when liquid medium was both on and beneath the plate was $3{\cdot}6 \pm 0{\cdot}3$ and when medium was beneath the plate only was $4{\cdot}1 \pm 0{\cdot}4$ ($P > 0{\cdot}4$). This result indicates that, at least in the case of intact flies in the presence of an odour stimulus, contact stimuli are not important.

2. A similar experiment was carried out with flies deprived of their antennae and palps. In this case twenty-two of each type of preparation were used. The mean number of egg masses when there was medium on and beneath that plate was $3{\cdot}3 \pm 0{\cdot}4$ and when medium was beneath only the mean was $1{\cdot}6 \pm 0{\cdot}3$ ($P < 0{\cdot}01$). This result indicates that there may be some contact effect when the fly is deprived

of the organs bearing most of its olfactory receptors (DETHIER, 1952). An alternative explanation is that the higher odour concentration due to the presence of the medium on the plate may have incited the flies to oviposit by stimulating odour receptors other than those on the antennae and palps. It can be concluded that contact chemical stimulation may play some role but that this is not significant when the fly is receiving sufficient stimulation from its olfactory receptors.

3. An experiment was done to demonstrate the role of an odour stimulus in oviposition. Six plastic containers each containing six females were set up with medium beneath the plate and a drop of 0·1 M sucrose solution on it. The oviposition in these was compared with that in another six containers in which there was no medium present. The mean number laid in the presence of the medium was $3{\cdot}0 \pm 0{\cdot}4$ egg masses per preparation whereas in the absence of medium the mean was only $1{\cdot}0 \pm 0{\cdot}2$ ($P < 0{\cdot}01$).

In a similar experiment the amount of oviposition when water was beneath the plate was compared with the amount when there was nothing beneath. The mean number of egg masses when water was beneath was $1{\cdot}4 \pm 0{\cdot}2$ per preparation and with nothing beneath was $1{\cdot}5 \pm 0{\cdot}4$ ($P > 0{\cdot}4$). It is clear from these results that the odour of the medium incited the flies to oviposit.

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B. *The loci of the receptors involved*

1. *Sensilla on antennae and palps.* Intact females and females which had had their antennae and palps removed were confined in plastic oviposition containers which had liquid medium beneath the plate and nothing on it. Seventeen preparations each containing six intact females and seventeen containing females deprived of their antennae and palps were used. The intact females laid $2{\cdot}8 \pm 0{\cdot}2$ egg masses per preparation and those without antennae and palps laid $0{\cdot}4 \pm 0{\cdot}2$ ($P < 0{\cdot}01$).

The possibility that operational shock may have caused the decrease in oviposition was excluded by the results of two experiments. In the first of these the amounts of oviposition by intact flies and by flies deprived of one antenna and one palp were compared. The mean number of egg masses laid by the intact flies was $3{\cdot}9 \pm 0{\cdot}4$ per preparation and that for the flies which had had the unilateral operation $4{\cdot}5 \pm 0{\cdot}4$ ($P > 0{\cdot}4$). In the second experiment flies which had had their antennae and palps removed immediately before the experiment were tested against flies similarly operated 24 hr earlier. Once again the numbers of egg masses laid were almost identical, the mean numbers for preparation being $2{\cdot}5 \pm 0{\cdot}3$ and $2{\cdot}3 \pm 0{\cdot}3$ respectively ($P > 0{\cdot}5$).

These results show clearly that at least some of the receptors involved in the reception of the odour of the medium are on the antennae or palps or both. No effort was made to evaluate the relative importances of the receptors on these organs but it is almost certain that the antennae are the more important loci.

2. *Sensilla other than those on the antennae and palps.* (a). The effect of the odour of the medium on the oviposition of flies which had been deprived of their antennae and palps was next examined. Six females without antennae and palps

were placed in each of sixteen plastic oviposition containers. In eight of these there was medium beneath and a drop of 0·1 M sucrose solution on the plate whereas, in the other eight, medium was absent. In the presence of the medium the oviposition rate was $2{\cdot}9 \pm 0{\cdot}3$ egg masses per preparation as compared with only $1{\cdot}0 \pm 0{\cdot}2$ in the preparations from which the medium was omitted ($P < 0{\cdot}01$). A similar test in which water beneath was tested against nothing showed that water itself has no effect; the respective means for eight trials of each being $1{\cdot}4 \pm 0{\cdot}3$ and $1{\cdot}5 \pm 0{\cdot}3$ egg masses per preparation ($P > 0{\cdot}8$). These results show that the flies are incited to lay by the stimulation of some receptors other than those on the antennae and palps by the odour of the medium. There is a possibility that the nerves exposed at the base of the antennae and palps by the removal of these organs may have been stimulated. But this is considered unlikely on the grounds that the exposed nerves would probably be damaged by desiccation and that a number of workers have successfully eliminated responses by the removal from insects of organs bearing sensilla (e.g. FRINGS, 1941; DETHIER, 1952). It seems more reasonable to interpret these results as indicating that odour receptors exist at other places on the fly. The results of experiments described below support the view.

(b). FRINGS (1941) showed that there are olfactory receptors on the labellum in the blowfly, *Cynomia cadaverina*. Attempts to determine whether sensilla on the labellum were involved in the reception of the medium odour by *P. regina* were unsuccessful because the oviposition rate of females deprived of their antennae, palps, and labella and those deprived only of their antennae and palps were very low in all of a large number of tests.

An experiment was done to determine whether flies deprived of their antennae, palps, and labella were stimulated to oviposit by the odour of the medium. Female flies deprived of all these organs were placed in plastic oviposition containers in the presence and absence of medium beneath the plate. Again the oviposition rates were found to be very low in most of the tests. In one experiment, however, in which there were six preparations with medium beneath the plate and six with only water beneath, there was satisfactory egg laying. In this the mean number of egg masses per preparation was $4{\cdot}0 \pm 0{\cdot}9$ in the preparations with the medium beneath and was only about $1{\cdot}0 \pm 0{\cdot}8$ in those with water beneath ($P < 0{\cdot}01$). Thus the presence of medium incited flies deprived of all the organs of the head known to bear olfactory receptors to oviposit.

(c). Six females which had had their antennae, palps, and labella removed were placed in each of sixteen oviposition containers in which the lower petri dish was divided in half by a vertical partition which sealed the two halves from one another. Medium was provided in one half and water in the other. A second petri dish containing medium was below the divided one so that there was little colour difference between the two sides. Medium was also present on the surface of the plate. In these sixteen tests, the holes with medium beneath them received a total of nineteen egg masses while the holes with water beneath received only one small mass. This result indicates that, even when deprived of all the organs of the head known to bear olfactory receptors, the flies were able to discriminate between the

two sides of the apparatus. There is indirect evidence that the site of these remaining receptors is the ovipositor. In the above experiment, it would be expected that the odour concentration above the plate would have depended largely on the distribution of medium on the upper surface and that those beneath the plate would have depended on the distribution of medium in the divided petri dish. Hence the difference in odour concentration between the two sides of the apparatus would be less marked above the plate than beneath it. The clear-cut distribution of egg masses in the apparatus indicated that, probably, the concentrations beneath the plate were important in determining where eggs would be laid. Since the ovipositor was the only part of the fly which was inserted through the holes it seems fairly certain that the ovipositor carried the sensilla involved.

This assertion is further supported by observational evidence. Female flies, whether intact or deprived of the olfactory sensilla on the head, when incited to extend the ovipositor and carry out probing movements on the surface of the plate, probed apparently randomly until the ovipositor came within a few millimetres of a hole over medium. At this point, the ovipositor, apparently moving independently of the rest of the fly, would turn toward the hole and would be unerringly placed through it. The fact that the ovipositor, rather than the whole fly, is involved in the oriented movement is taken as additional evidence for the presence of olfactory chemoreceptors of the ovipositor. Finally, there is the morphological evidence that there are typical olfactory sensilla on the lateral valves of the ovipositor. There is little doubt that the ovipositor bears sensilla which enable the fly to make directed movements with its ovipositor towards the source of the odour. Efforts to remove or cover the lateral plates were unsuccessful. The flies so treated did not lay eggs even when otherwise intact.

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DISCUSSION

The results show clearly that the sensilla chiefly involved in the reception of odours which stimulate *P. regina* to oviposit are situated on the antennae or palps or both. DETHIER (1952) has shown that the sensilla concerned with directed locomotor responses of this fly to olfactory stimuli are located on the antennae and palps. CRAGG and COLE (1956) obtained similar results with *Lucilia sericata* and *L. cuprina*. Thus the sensilla involved in the two types of responses have similar loci. From the evidence available it is not possible to tell whether the same receptors are involved in each reaction. If the same receptors were concerned it would be expected that all volatile oviposition stimulants would also be attractants and that all volatile deterrents would also be repellents.

The presence of chemoreceptors on the antennae, mouth parts, and tarsi of insects is well known but there have been only a few demonstrations of their presence on the ovipositor and these have dealt almost entirely with rejection. DETHIER (1947) showed that the ovipositor of the wasp *Nemeritis canescens* is sensitive to solutions of certain salts and aliphatic alcohols and that rejection occurs. Evidence that chemoreceptors exist on the ovipositors of other hymenopterous parasites is summarized by DETHIER (1957). The ovipositor of *Gryllus assimilis*

has been shown to be sensitive to alcohols (DETHIER, 1951) and sodium chloride (DETHIER, 1956). Electrophysiological evidence that sensilla on the ovipositor of *P. regina* are stimulated by sodium chloride has been provided by WOLBARSHT and DETHIER (1958). The evidence for the existence of olfactory receptors on the ovipositor of *P. regina* given in this paper is the first which suggests that the stimulation of sensilla on the ovipositor may play a part in inciting an insect to oviposit.

It is not possible to elucidate with any certainty the specific roles in oviposition of stimulation received by way of the sensilla on the antennae and palps and that received by way of those on the ovipositor. It seems likely, however, that stimulation of the sensilla on the antennae and palps causes the fly to extend its ovipositor and that only then do those on the latter play an important role. Probably the information received from the receptors on the ovipositor enables the fly to place its eggs accurately at the point where the concentration of the oviposition stimulant is optimal.

Acknowledgements—I wish to thank Professor V. G. DETHIER, Mr. G. W. GREEN, and Dr. D. R. EVANS for the many helpful suggestions they made during the course of this work.

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STUDIES ON THE RELATIONSHIPS BETWEEN AIR TEMPERATURE AND THE INTERNAL BODY TEMPERATURE OF *LOCUSTA MIGRATORIA*

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Abstract—Changes in the body temperature of *Locusta migratoria* L. when the air temperature around it was slowly increased have been recorded. The responses found varied according to the physiological condition of the insect. In normal insects the body temperature showed an initial lag of about 1·4°C behind that of the air. After the first 15 min the body temperature increased at the same rate as the air temperature. When changes in air temperature were stopped, the body temperature still increased and showed a temperature excess of about 0·5°C above that of the air. Experimental technique precluded the possibility of this being due to radiant heat, exercise, or conduction from hotter objects. Normally, the locusts were brought into equilibrium with a temperature of, or just above, 20°C. If, however, they were equilibrated with a lower temperature (15°C), then the response curve showed a greater lag lasting for a longer time followed by a body temperature increasing more rapidly than the still increasing air temperature. The final overshoot was not so large and was present in only 60 per cent of the animals tested.

Starved locusts were incapable of increasing their body temperatures at a rate greater than that of the air and showed a continually increasing depression until the air temperature was held steady. There was no overshoot at the end of the experiment. Injections of sub-lethal doses of potassium cyanide had a similar but less severe effect. Injections of 2 : 4–dinitrophenol (DNP) did not increase the body temperature in a constant environment of 20°C. It had a most marked effect when the air temperature was increasing, the response curve immediately after the initial depression showing a rate of increase in body temperature in excess of that of the air. The body temperature actually exceeded that of the air whilst the air temperature was still increasing. The final overshoot was about 1°C.

The possible causes of these different response curves are discussed. It is thought that they cannot be explained on the assumption that body temperature depends solely upon the absorption of heat from the air with its consequent stimulation of metabolism. But, that the air temperature through the stimulation of temperature receptors causes the release of substances into the haemolymph which stimulate metabolism, and that part of the increase in body temperature results from this. The mechanism may be important in that it enables the insect to reach equilibrium conditions with its environment more quickly than it could in its absence. It also enables the insect to speed up its development by allowing the maximum use to be made of transient increases of air temperature themselves too feeble to warm the mass of the locust.

INTRODUCTION

In the past, studies upon the relationship between internal body temperature of an insect and air temperature have paid little attention to the physiological condition of the insect and the effect this may have on the events recorded. This is probably due to the obvious importance of physical factors such as evaporation,

wind speed, and radiant heat in determining the broad course of these relationships. Thus the studies of PIRSCH (1923), NECHELES (1924), and KOIDSUMI (1934) have shown that, in the absence of radiant heat, the body temperature of an inactive insect is higher than that of the air in its sub-optimal zone and lower in its supra-optimal zone, providing that the air is dry, indicating that evaporation of water is the key factor. BODENHEIMER (1929) and KOIDSUMI (1934) have shown that when an insect is suddenly transferred to a lower or higher temperature the curve expressing the change in body temperature is similar to that for Newton's law of cooling. This, together with observations by HUSSEIN (1937) that the time taken for temperature changes to influence the behaviour of locusts is greater for larger insects than for smaller ones, suggests that loss or gain of heat from the air is a simple physical phenomenon.

In the presence of a source of radiant heat there are many observations both from the field and the laboratory which show that the body temperature of the insect is in excess of that of the air. Calculations by PARRY (1951) and experiments by DIGBY (1955) show that this excess is proportional to the radiation strength, varied with the power of the size of the body, and, over a certain range, varied inversely with the square root of the wind speed.

In all these cases the magnitude of the changes imposed upon the insect would largely blanket any effect the physiological condition of the insect would make to the responses observed. During experiments in which the changes induced were of a somewhat gentler nature than those given above, it was found that the body temperature of the insect responded with a definite pattern which depended upon the pre-treatment of the insect and which indicates that the changes in the insect are by no means passively following those of the environment. It is the purpose of this paper to describe these changes.

MATERIAL AND METHODS

Treatment of the locusts

The insects used were all males of *Locusta migratoria* between 3 and 10 days old. The animals were reared at 28°C and 70 per cent r.h. They were subjected to a photoperiod of 12 hr light and 12 hr dark and always provided with abundant fresh food and water. Immediately before the commencement of each experiment animals were removed from these conditions and kept for 1 hr at a temperature of 22°C.

The insects were prepared for the experiment by having their wings held together with a small piece of Sellotape, and were fastened on to a perspex perforated platform by means of a strip of Sellotape over each leg. Secured in this manner, the majority of the animals had little tendency to struggle and observation showed that they sat quietly throughout the course of each experiment. Results from those which were observed to struggle are not used in this paper.

After the insect had been secured, a small hole was made by a scalpel in the right mesepisternum of each insect and the thermocouple inserted through this

hole until the polyvinyl sheath came into contact with the cuticle. The junction between the sheath and the cuticle was sealed by means of a small piece of plasticine. A large piece of plasticine which was used to hold the thermocouple in place did not touch the insect and stood some 5 mm away from it. Particular care was taken to see that there was no leakage of fluid around the thermocouple, the seal being inspected and found intact at the end of each experiment. The locust, mounted on the platform, was then inserted into the apparatus and left there for 30 min to equilibrate. At the end of this time temperature readings were taken every 5 min throughout the experiment. At the end of the experiment the animals were removed from the apparatus, freed from the thermocouple, and weighed, care being taken that no damage was done by the removal of the Sellotape. The insects were returned to the insectary and were alive at least 7 days later.

Tests made by inserting the thermocouples into the mesepisternum of locusts and then killing them and dissecting to see where the thermocouple lay, showed that in 80 per cent it lay under the crop in the mid-line of the body, in 15 per cent at the side of the crop, and in 5 per cent its precise position was not determined.

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Measurement of temperature

Air and body temperatures were measured by means of copper–constantan thermocouples made from S.W.G. 40 enamel-covered wire. The enamel was removed from the ends of the wire by means of steel wool and the tips of the wire cleaned with absolute alcohol. The wires were then twisted together and fused with a spot welder. This gave a small spherical junction about 0·5 mm in diameter and 1·2 mg in weight. The junctions were insulated with enamel and baked hard in an oven. Each pair of thermocouples was then mounted by sleeving the wires in fine polyvinyl tube to within 5 mm of the junction. The wires were held in the tubing by a drop of shellac. Each pair of thermocouples was arranged so that one member was held at constant temperature in a Sunvic Hot Junction. These will hold a temperature constant to better than 0·1°C, and experience showed that with careful packing a temperature differential of less than 0·05°C could be obtained for long periods. Differentials of this magnitude were held throughout the duration of the experiments reported below.

The potential developed by the thermocouples was measured by a Cambridge Slide Wire Potentiometer graduated in 2 μV steps and capable of being read to 1 μV. A Cambridge Spot Galvanometer giving 1 mm deflexion for changes in 1 μV of the potentiometer was used as a null point instrument.

The thermocouples were calibrated over the range 0–50°C against a high-quality mercury in glass thermometer graduated in 0·1°C, and over 5°C ranges at 15–20°C, 30–35°C, 40–45°C against a Beckman thermometer calibrated in 0·01°C. The potential developed by the thermocouples ranged from 39·92 to 39·89 μV/1°C. In calculations throughout this paper this is treated as 40 μV/°C and as if the potential over the range 15–40°C was linear. Error from both these assumptions was less than 1 per cent.

Apparatus used for changing the environmental temperature

A diagram of this apparatus is shown in Fig. 1. It consists of a closed circulatory system through which air is pumped at the rate of 2 l./min. This gives an air flow around the locust of 10·7 cm^3/min per cm^2. Air from the pump was heated by passing through a spiral immersed in the water bath, its speed measured

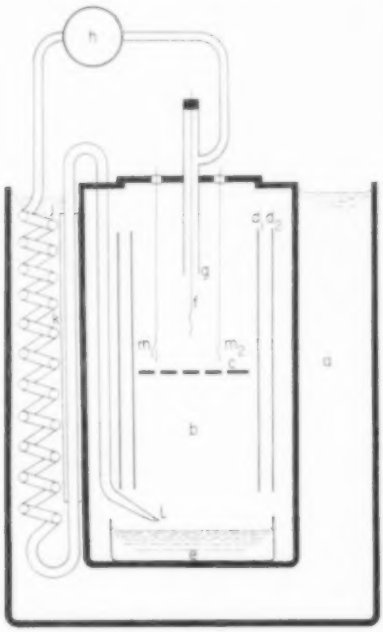

FIG. 1. Diagram of apparatus used to change the air temperature around the locusts. a, thermostatically controlled water bath; b, air chamber; c, perforated perspex platform; d_1 and d_2, radiation shields; e, vessel containing saturated solution of KCl; f, air thermocouple; g, air exit from chamber; h, air circulation pump; j, air heating spiral; k, rotameter; l, air input; m_1 and m_2, thermocouples to animal. For further information see text.

by a rotameter, and then passed into the experimental chamber to be discharged over a solution of saturated potassium chloride. Air was extracted from the chamber through a tube opening into its top centre region. The speed of the air flow was controlled by altering the speed of the pump.

The temperature of the water bath which was used to heat the air as well as the walls of the experimental chamber was controlled by a contact thermometer at the extremes of the temperature cycle, while change in temperature was brought about by three 100-W heaters working against a known volume of water. Although precise repeatability of rate of temperature change was not obtained by this method, differences of 0·1°C/min represent the extreme range in the recorded experiments. Measurements with a shielded thermocouple showed that the air

within the chamber was always at a slightly higher temperature than the walls. To exclude radiant heat copper radiation shields were placed around the locust. They were suspended from the perspex roof of the chamber by fine wires and were separated from each other and from the walls of the air bath by 1 in. Tests with blackened and bright unshielded thermocouples within these walls showed that the air temperature was always greatest and radiant heat was absent. Considerable changes in humidity with increase in temperature were prevented by the presence of a saturated solution of potassium chloride in the bottom of the experimental chamber. Humidity was measured by calibrated wet and dry thermocouples and showed a decrease of from 10 to 15 per cent in the course of an experiment.

EXPERIMENTAL RESULTS

The changes recorded for both air and body temperatures are shown in Fig. 2a. The rate at which the temperature increases, which was nearly the same in all cases, being $0{\cdot}18 \pm 0{\cdot}05$°C/min, tends to conceal the differences between these curves. The results are therefore plotted as the differences between air and body temperature (Fig. 2b). The times at which the air temperature increased and at which it was stopped are marked by vertical lines on the graphs. As the response of the animal differed according to its prior treatment, it is convenient to classify the results according to the treatment applied.

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1. *The responses of normal adult locusts*

Normal locusts are those taken from the stock culture, and all were more than 5 days old but not yet sexually mature. The results obtained from them are shown in Fig. 2b. The general pattern of these curves showed an initial depression during the first 15 min of temperature change. Reference to Fig. 2a shows that the absolute body temperature of the locust increased during this time, but less quickly than that of the air. Once this period had passed, the body temperature increased approximately as rapidly as that of the air. In a few cases it increased somewhat more rapidly, although the body temperature was always less than that of the air. When the air temperature change was stopped and remained constant, the body temperature still increased, the final temperature being 0·19–0·48°C in excess of that of the air.

The pattern of these curves suggests that the locust does not depend entirely upon heat absorbed from the air in order to bring about an increase in its body temperature. Three features support this point of view. One: after the initial depression the changes in body temperature keep pace with those of the air; if absorption of heat from the body surface was relied upon, the value of the depression should increase for as long as the environmental temperature is increased. Two: there is no positive correlation between the surface area or weight and the maximum temperature depression of the insect found in each case. Three: there is an overshoot of body temperature above that of the air after the changes in air temperature have ceased. This overshoot can be taken as direct evidence of active adjustment on the part of the locust to changing air

temperatures. It is worth noting that at the upper temperature the relative humidity was only 70 per cent, which provides suitable conditions for the insect to cool itself by the evaporation of water; even so an excess of temperature is found. The possibility that this was due to radiant heat from objects surrounding

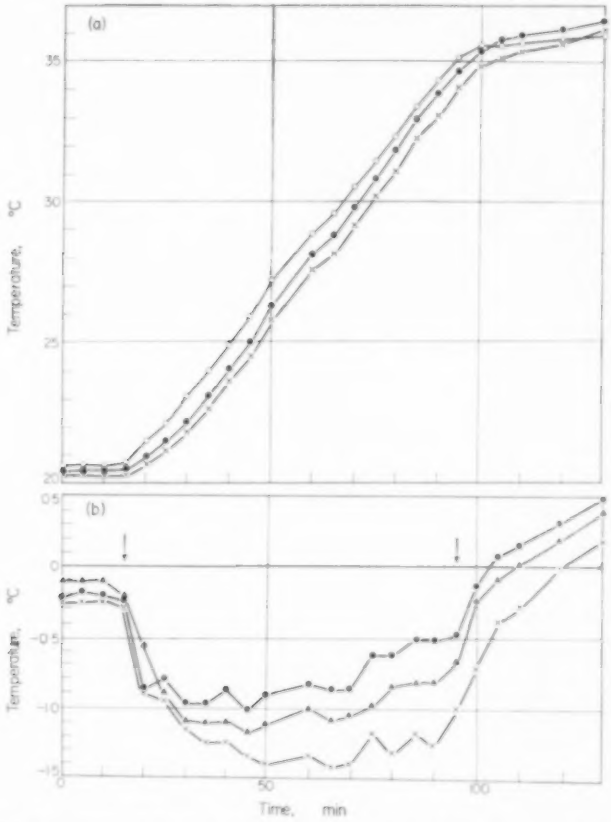

FIG. 2. The changes of body temperature of normal adult locusts with increasing air temperature. (a) Changes in air and body temperature against time. ○ —— ○ air temperature; ● —— ●, × —— ×, changes in body temperature. (b) The changes in the difference of air and body temperature with time. The differences plotted are between the curves shown in (a), together with one other curve omitted from (a).

the locust can be rejected for two reasons: the metal radiation shields were at a slightly lower temperature than the air, and under identical experimental conditions this overshoot can be abolished by pre-treatment of the locust.

Further evidence that this was an active process was obtained by commencing the experiment at a lower starting temperature than usual. The normal active range of the animal has a lower limit at 20°C, and all but the next set of experiments were started at this or a slightly higher temperature. If, however, the

experiment is done on an animal in equilibration with a temperature of 15°C, a different form of response curve is obtained (Fig. 3). The period of depression lasts until half-way through the experiment, and reaches a value of 3°C, which is greater than that previously recorded. The body temperature then increases

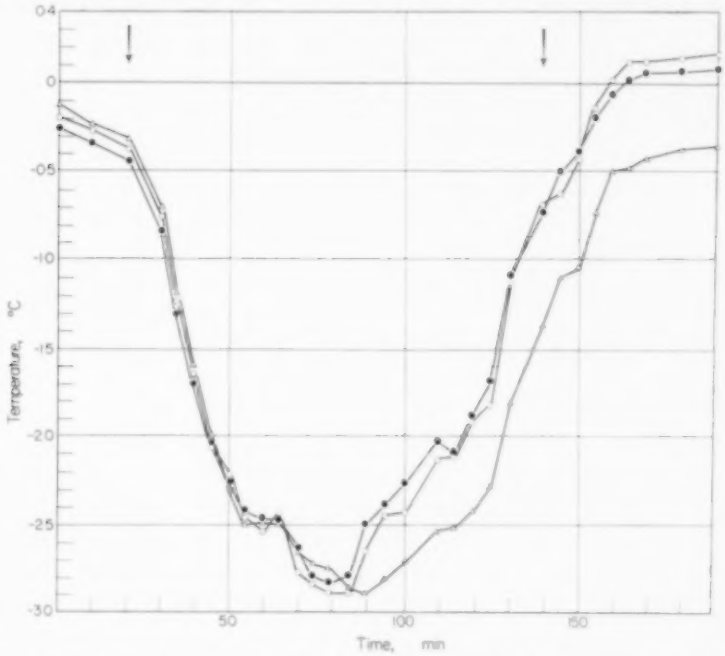

FIG. 3. The difference between air and body temperature with time of normal adult locusts when the range of temperature change was between 15 and 36°C.

in comparison with the air temperature; the final overshoot, however, is not so large as in the previous experiments, and occurred only in about 60 per cent.

As the rate of temperature change was the same in this as in the previous experiments, there seems no reason why, if surface absorption of heat is the sole factor in body temperature increase, the temperature should not have followed a similar course to that previously described. It seems probable that, as 15°C is less than the normal range of the animal, the differences may be due to failure of the compensatory system to function outside the normal range of the animal, and the smallness or lack of overshoot to the limited capacity of the system, which, however, must have been quite active to produce the increase in body temperature observed.

2. *The responses of starved animals*

These observations were conducted upon adult locusts of the same age group as previously, but which had been kept without food or water for 48 hr before

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the experiment. The results are shown in Fig. 4. The curve shows a relative decrease in body temperature lasting for about 20 min; this is followed for a similar period by the body temperature remaining steady at 1·4–1·5°C less than that of the air; then there is a further period of decrease lasting until the air temperature ceased to change, the final depression being about 2·2–2·3°C less

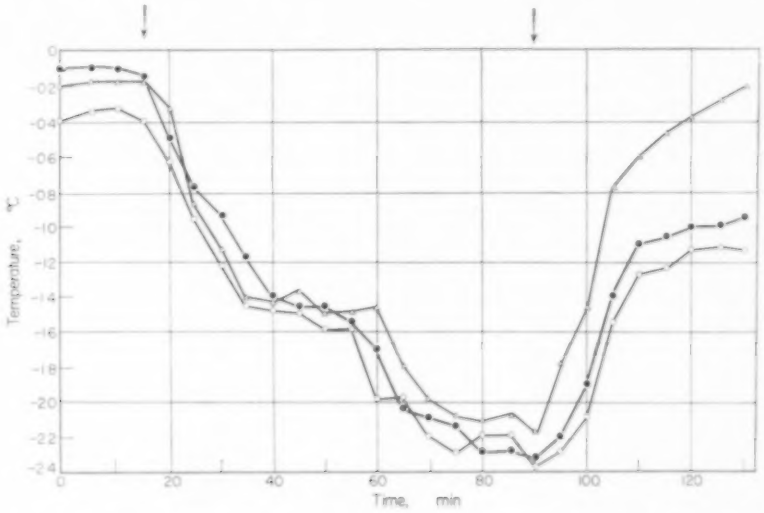

FIG. 4. The difference between air and body temperature with time in adult locusts which had been kept without food and water for 48 hr prior to these observations.

than that of the air. With the cessation of increase in air temperature the body temperature increased rapidly; there was, however, no overshoot, and at the end of the experiment the body temperature was considerably less than that of the air.

In general, this curve is more in keeping with that which might be expected if the animal were solely dependent upon the absorption of heat through its surface for increasing its body temperature. The presence, however, of a short steady period indicates that the locust is not entirely passive in this matter. In any case, the starved locust does not bring itself into equilibrium with changing air temperature in the same manner as does a fully fed one. It is apparently more dependent upon the physical factors of the situation, and this supports the suggestion that the fully fed locust actively responds to changes in air temperature.

3. *The effect of drugs*

All animals used in these experiments were normal adult males of the age-group 5–10 days, taken just before the experiment from the stock cages. After the experiments they were returned to the insectary and all were found to be alive and well at least 4 days later. The purpose of using drugs was twofold:

to estimate to what extent the locust could alter its body temperature by metabolic activities; and to use a depressant, which if metabolic heat plays no part in temperature change should not affect the normal changes in body temperature already described.

The drugs used were 1/100 M potassium cyanide as a metabolic depressor, and 1/1000 M 2 : 4-dinitrophenol (DNP) as a metabolic stimulant. For each experiment three animals were used: one as a control was injected with 20 μl of distilled

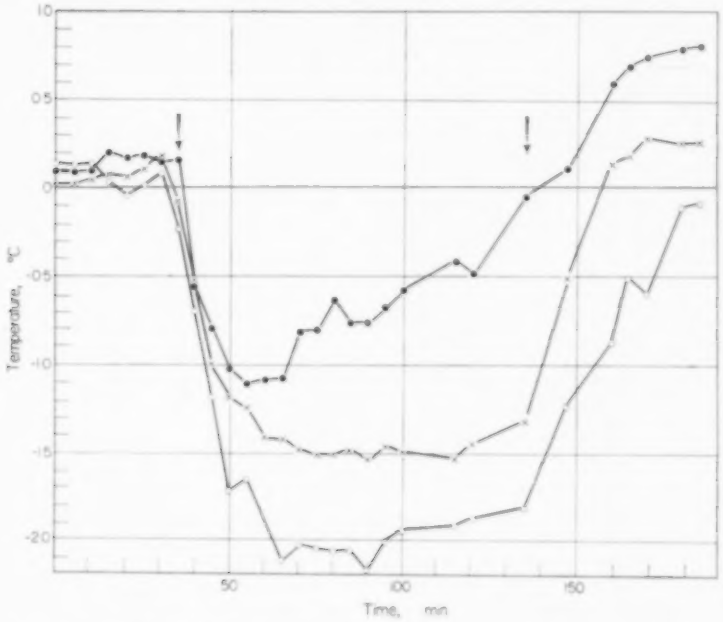

FIG. 5. The difference between air and body temperature with time, in normal adult locusts that have been injected with drugs immediately prior to these observations. ×——× control injected with distilled water; ○——○ injected with KCN; ●——● injected with 2 : 4-dinitrophenol (DNP). For further information see text.

water, one injected with 20 μl KCN, and one with 20 μl DNP. Each experiment was repeated three times, and for each one locusts as nearly as possible of the same weight were used so that size differences would not interfere with their comparison. Results of this experiment are shown in Fig. 5.

The control animal injected with water showed an exactly similar response to that found in normal uninjected locusts. Those injected with KCN showed a greater initial depression than normal, after which events were similar to the control until the time of cessation of air temperature change, where the rate of recovery was slightly slower and there was no overshoot, the final body temperature being a little below that of the environment.

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The locust injected with DNP showed a smaller temperature depression than the control, and this was followed immediately by a rate of increase in body temperature greater than the rate of increase of air temperature. In general

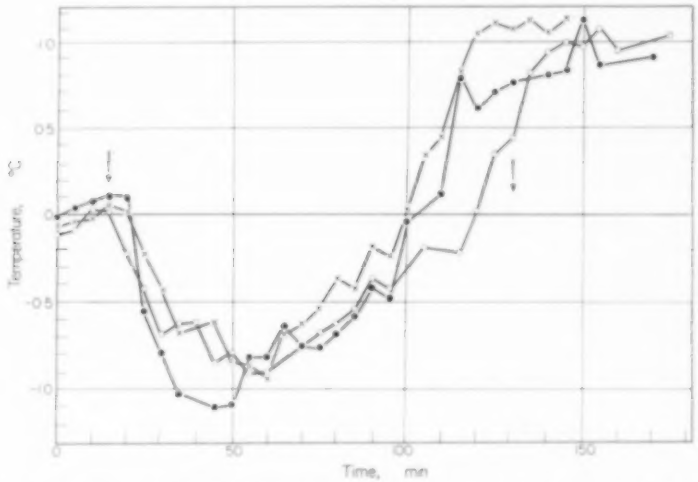

FIG. 6. The difference between air and body temperature with time in normal adult locusts injected with DNP when the air temperature is increased.

this was so great that the body temperature became greater than the air temperature, although the latter was still increasing (Fig. 6). This rate continued until an overshoot of approximately 1·0°C occurred and was maintained until the end of the experiment.

This experiment shows again quite clearly that increase in the animal's metabolism plays an important part in the response the locust makes to changing air temperature, since its normal pattern can be altered by the use of metabolic inhibitors and increased by metabolic stimulants although the environmental circumstances are identical in each case.

It seemed advisable to test the effect of the DNP at constant temperatures as well as increasing temperature (Figs. 6 and 7). The experimental procedure used was the same as previously, but, of the three animals used, one was used as control and two given the experimental injection. The effects were observed at 20°C and at 30°C.

At 20°C (Fig. 7) the drug showed no stimulatory effect over a 3-hr period. The dip in the curve at 15 min represents the temperature change encountered when the animal was removed from the apparatus for injection. All three animals remained very close to the air temperature and there was no difference between injected and control animals.

At 30°C some stimulatory effect was found. This effect upon body temperature was, however, very much less than that found when the air temperature was

changing. The temperature excess produced was 0·25°C above that of the control animal, whereas the temperature excess of an injected over a control animal under changing environmental conditions was 0·55–0·8°C. The experimental

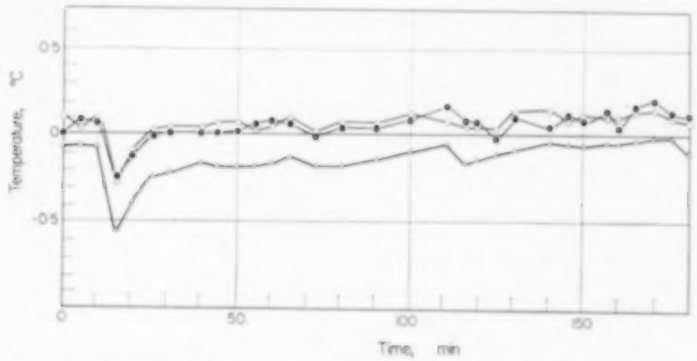

FIG. 7. The differences in air and body temperature with time in normal adult locusts that have been injected with DNP when the air temperature is kept constant. ●——●, control injected with distilled water; ○——○, △——△ locusts injected with DNP.

conditions for 30°C allowed a rather greater stimulus of temperature to be applied when the animals were removed for injection than when they were at 20°C. All animals are thought to have shown the stimulatory effect as a result of this.

DISCUSSION

As there have been few experiments done by other workers along the lines indicated in this paper, there is little to which the results obtained can be compared. Both BODENHEIMER and KOIDSUMI changed the temperature over their animals abruptly and through a larger range. Some of their curves, if extrapolated, show that the body temperature would have been in excess of air temperature, but unfortunately, they stopped their measurements at the equilibration point. Their experimental data, unlike that presented here, does not preclude the possibility of radiant heat being responsible for this excess. Extensive measurements of air and body temperature in locusts have been made by KENNEDY (1939). These show that for any given air temperature the body temperature may be anywhere within a range of ±4°C, the temperature excess recorded in this paper falling well within this range.

There is a considerable amount of work which suggests that the metabolism of an insect is influenced by the temperature of its environment (UVAROV, 1931; WIGGLESWORTH, 1950; ROEDER, 1953). There is no discussion on how this stimulation may be brought about. The underlying assumption in all cases seems to be that the air warms up the body, increasing its temperature and thus speeding up the chemical reactions of metabolism. The curves obtained above suggest that the sequence of events may not be as simple as this, and that the insect

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reacts in a more positive fashion to changes in air temperature. This is the principal conclusion drawn from the experimental evidence given above and it rests on the following observations.

There is no positive correlation between the weight of the locust, its surface area (calculated from the weight by the formula given by SLIFER, 1954), and the maximum temperature depression recorded. Such a correlation should exist if the increase of the body temperature of the insect were solely dependent upon conduction of heat from the air to the body.

In locusts of the same instar and weight, the manner in which the body temperature increases with increasing air temperature depends upon whether the insect is 'normal' (Fig. 2), whether it has been starved (Fig. 4), or if the starting temperature is 15 or 20°C (Figs. 2 and 3). These differences would not be encountered if the increase in body temperature rested only on the absorption of heat from the air unless the metabolism was contributing to the increase of body temperature.

The form of the response curve could be changed by injecting the locust with dilute solutions of potassium cyanide. Locusts injected with an equal amount of distilled water showed normal response curves. Comparison of potassium cyanide injected and normal locusts shows to what extent the metabolism contributes to increasing the body temperature. Potassium cyanide injected animals show an increased lag in the time before body temperature starts to increase, and they do not show a temperature excess at the end of the experiment (Fig. 5).

The response curve is altered from that found in normal locusts by an injection of 2 : 4-dinitrophenol (DNP). DNP is known to stimulate heat production in animals by 'uncoupling' oxidative phosphorylation, the energy normally used in the synthesis of high-energy phosphate bonds being dissipated as heat. It is not therefore surprising that it causes the body temperature to increase. What is surprising is that this only happened when the air temperature around the animal was increasing (Fig. 6); when the air temperature was held constant at 20°C no increase in body temperature resulted from the injection of DNP (Fig. 7). At 30°C constant temperature some stimulation was found, but the experimental technique was such that the locust suffered a temperature change of some 3°C when it was removed from the apparatus for injection. This change may have been enough to produce the required stimulation. At 20°C little change in temperature occurred when the locust was removed for injection. Further, heat production by injection of DNP is more effective at low than at high temperatures (SOLLMANN, 1948).

In all normal locusts tested the body temperature was found to continue to increase after the air temperature had ceased to change, and at the end of the experiment to show an excess of about 0·5°C above that of the air. In the experiments at which the animal was in equilibrium with a lower temperature (15°C instead of 20°C) at the commencement of the experiment, the body temperature was found to be increasing faster than the air temperature over the latter part of

its range, and in some cases actually to overtake the air temperature whilst that was increasing. Observations showed that this could not be due to radiant heat, visible struggling by the locust, or conduction from hotter objects in contact with the insect. It could only be due to heat generated by increased metabolism. Since over certain parts of the curve the body temperature was in excess of the still increasing air temperature, the latter could not be affecting the former by the diffusion of heat from the air into the body, and its stimulatory effects must be accounted for in some other way. It is important to note that no claim is made about the relative importance of this to other factors such as radiant heat that may play a part in determining the body temperature of the locust, only that the part played by metabolism can be demonstrated under appropriate conditions.

As a hypothesis it is suggested that an increase in air temperature stimulates the temperature receptors of the insect, and these mediate the release of some substance into the haemolymph which stimulates metabolism, either generally, or particularly those actions which generated most heat. This would result in the body temperature increasing more rapidly than if conduction of heat from the air was the only means of warming the body.

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A possible explanation for the existence of such a mechanism is that insects are too small to maintain a temperature different from that dictated by their surroundings without the expenditure of a disproportional amount of energy. To maintain a temperature excess by exercise or metabolism would require a high food intake; whilst cooling, requiring loss of water or high tracheal ventilation rates, also has its limiting factors. Judging from the work of KROGH (1914), there is no optimum temperature at which the energy required to complete a developmental stage is at a minimum. Providing completion is possible at a given temperature, the total energy expended would be the same at all temperatures. This would not be true if some energy has to be expended in maintaining a temperature different from that of the environment: therefore, an identity of body temperature with that dictated by its surroundings represents the most economical situation. If the environmental temperature changes, then the quicker the insect can bring itself into equilibrium with the new conditions the less energy it will waste in opposing them. Such a mechanism as I have proposed would aid in this. The speed of development is also an important aspect affected by temperature. If metabolism can be stimulated through temperature receptors, the greater the use the insect can make of transient temperature increases the faster it will grow. That locusts do grow faster under cyclical conditions has been demonstrated by HAMILTON (1936).

SUMMARY

(1) The response curve for changing body temperatures induced by changing air temperatures varies with the physiological condition of the locust.

(2) In normal locusts the curve after an initial depression keeps pace with slow changes in air temperature, and when the air temperature ceases to change,

the body temperature goes on increasing, having finally a temperature excess of 0·2–0·5°C above that of the air.

(3) In starved locusts, depression of the body temperature continues throughout the whole period that the air temperature is increasing, and at the end of the experiment there is no overshoot of body temperature above that of the air.

(4) Injections of sub-lethal doses of potassium cyanide also resulted in the response curve being depressed from its normal course and in preventing overshoot at the end of the experiment.

(5) Injections of 2 : 4-dinitrophenol caused a rapid increase of body temperature when the air temperature was increased. The body temperature showing an excess over the air temperature whilst the latter was still increasing, and the overshoot at the end was high (ca. 1°C).

(6) Injections of DNP failed to stimulate the metabolism when the air temperature was kept constant.

(7) It is suggested that increases in body temperature dependent upon metabolism are not mediated by the absorption of heat through the body surface of the locust, but that the metabolism is increased by a substance released into the haemolymph, the release of the substance resulting from the stimulation of temperature receptors by increasing air temperature.

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THE EFFECT OF INSECT HORMONES AND OF 2:4-DINITROPHENOL ON THE MITOCHONDRION OF *LOCUSTA MIGRATORIA* L.

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Abstract—Mitochondria were prepared from the fat body and thoracic muscles of *Locusta migratoria* and *Schistocerca gregaria*. The respiration of the mitochondria in a sodium succinate substrate was measured, and the effects of adding various substances on the oxygen consumption of the mitochondria recorded. The addition of 2 : 4-dinitrophenol (DNP) in physiological quantities resulted in a decrease in oxygen consumption when compared with a control on mitochondria prepared from adult *Locusta migratoria*. It increased the oxygen consumption of mitochondria from the fifth instar of *Schistocerca gregaria*.

The addition of the corpora allata to mitochondria prepared from adult *Locusta migratoria* caused an increase in oxygen consumption whilst their addition to those prepared from fifth instar *Schistocerca gregaria* decreased oxygen consumption. When both DNP and corpora allata were present together the oxygen consumption was stimulated in both *Locusta* and *Schistocerca*. The amount of stimulation was slightly greater than when either was present alone. The effect of the addition of the corpora cardiacum to preparations of adult mitochondria was to depress their oxygen consumption. One of the sites of the action of the corpora allata hormone is shown to be the mitochondrion. The possibility that the known action of DNP might provide a further clue to the site of action of the C.A. hormone is discussed.

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INTRODUCTION

In a previous paper it has been shown that the change in body temperature resulting from a change in environmental temperature in a locust depended upon the physiological condition of the insect, and not solely upon the absorption of heat from the environment. Also, it proved impossible to raise the body temperature with 2 : 4-dinitrophenol (DNP) when the air temperature was kept constant, although a considerable excess of body temperature over that of the control locusts was obtained when the air temperature was slowly increased. One of the sites of action of DNP is the mitochondrion. It was therefore decided to take advantage of opportunities available to prepare mitochondria from the thoracic muscle and fat body of *Locusta migratoria* L. and *Schistocerca gregaria* Forsk., and test the effect of this substance upon them. Since it was also found that some stimulation was obtained under certain circumstances in the absence of DNP, the effect of insect hormones was also tested on the mitochondria.

Unfortunately the necessary equipment for the preparation of mitochondria was only available for a short time, and only seven experiments could be performed.

The results obtained from these were judged to be of sufficient interest to publish, although much more work is clearly necessary to place the conclusions suggested upon a firm basis.

MATERIAL AND METHODS

The locusts used in these experiments were adults of both sexes of *Locusta migratoria* L. and fifth instar larvae of *Schistocerca gregaria* Forsk. The locusts were reared and kept at 28°C and 70 ± 5 per cent relative humidity, and exposed to a 12-hr light/dark photoperiod. Abundant food and water was always available to them. The locusts were removed from these conditions immediately prior to the experiment.

Removal of tissues from the locusts

Fat body was obtained from the locusts in the following manner. The locusts were decapitated and the body opened in the mid-ventral line cutting from the prosternum to the tip of the abdomen. The gut was turned back, the gonads removed, the fat body gathered together with a pair of forceps and immediately transferred to a tared weighing bottle standing in crushed ice.

Muscles were now obtained from the thorax by cutting through the longitudinal dorsal flight muscles where they joined their phragmata; the tergo-sternal muscles, the direct flight muscles and the coxal muscles were freed from the exoskeleton by cutting through their attachments medially and laterally. This left a mass of loose muscle which could be picked up by forceps and was again transferred to a tared chilled weighing bottle. With a little practice it was found that the time taken to perform both these operations was a little less than one minute, so it was unnecessary to cool the locusts whilst these dissections were being performed.

Preparation of mitochondria

The methods used for the preparation of mitochondria were similar to, and based on, those used by BELLAMY (1958), all operations being carried out as close to 0°C as possible.

Homogenization

The tissue was homogenized in an ice-cold, all-glass, Potter–Elvehjem type homogenizer with 4 vol of medium (0·25 M sucrose adjusted to pH 7·6 with 0·1 M 2-amino-2-hydroxymethyl-propane-1 : 3-diol) using 20 passes at 5000 rev/min. The homogenate was then diluted with a further 5 vol of medium prior to centrifugation.

Centrifugation

Cell debris and nuclei were sedimented by centrifuging the homogenate three times at 900 *g* for 10 min, the residue being discarded each time. Large quantities of yellow fat were encountered in homogenates of fat body, which on

centrifugation rose to the surface. Providing centrifugation was carried out between 0 and 2°C, much of this material could be removed by carefully withdrawing the supernatant layer from underneath it with a capillary pipette.

Mitochondria were sedimented as a brown pellet by centrifuging at 10,200 *g* for 10 min and the cloudy supernatant was discarded. The pellet was then washed twice by re-suspension in 10 vol of fresh medium, the last centrifugation being carried out at 25,000 *g* for 10 min. Wet weight yields which are given in Table 1 averaged 14 per cent for fat body and 7 per cent for muscle. The final mitochondrial pellet was re-suspended in ice-cold sucrose medium and used immediately for respiratory determinations.

Determination of oxygen consumption

The measurement of oxygen consumption was carried out in a standard Warburg apparatus, the volumes of the flasks being approximately 6 ml, the gas phase air, the temperature 30°C, and the equilibration time 10 min; the period of observation was 1 hr.

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The incubation mixture contained 10 μmoles of sodium succinate, 2 μmoles of magnesium chloride, and 0·01 M potassium phosphate buffer, pH 7·4, and was adjusted to 0·25 osmolar with sucrose solution. The tissue suspension (0·5 ml) was added to give a total fluid volume of 1 ml.

Where necessary 0·04 M 2 : 4–dinitrophenol (0·25 μl) was added to give a final concentration in the manometer flasks of 10^{-4} M.

Preparation of the hormones

The hormones used were obtained by dissecting the appropriate organs from mature adult female *Locusta migratoria*. As short a time as possible before the mitochondria were ready (20 min) the locusts were taken from the insectary, beheaded, and the head capsule split in the mid-line from the foramen magnum to the median ocellus. It was then opened and held open by the insertion of a pair of forceps between the compound eyes. The posterior part of the brain was thus readily made visible, and with a little dissection the corpora allata and the corpora cardiaca easily found. These organs, together with the protocerebrum, were removed and kept in chilled watch glasses on crushed ice until used. Microscopic examination showed no change in the organs from the moment they were removed from the body until the time they were placed in the flasks.

After the incubation mixture and the mitochondria had been added to the flasks the endocrine organs were removed from the watch glasses by means of a glass rod, placed in the incubation mixture and ground up against the wall of the manometer flask.

RESULTS

Details of the experiments are given in Table 1. The effect of 2 : 4–dinitrophenol on the oxygen consumption of mitochondria is shown in Figs. 1 and 3. In all cases the oxygen consumption was inhibited by the presence of DNP. This is

TABLE 1—THE EFFECT OF DNP AND OF INSECT HORMONES ON THE RESPIRATION OF MITOCHONDRIA

Expt.	No. of locusts	Yield of mitochondria wet wt. (%)	Wet wt. of mitochondria added to flask (mg)	Treatment	Oxygen consumption O_2/hr/100 mg wet wt.
			(a) Mitochondria from fat body		
Adult *Locusta migratoria*					
1	33	12	16	Control	14·51
					14·13
				+ DNP	8·35
					9·22
2	34	14	24	Control	29·11
				+ C.A.	33·36
				+ DNP	25·37
3	35	12	250	Control	31·07
				+ C.A.	36·42
				+ DNP	22·04
				+ DNP and C.A.	38·85
4	12	20	15	Control	67·42
				+ C.A.	76·92
				+ DNP	59·79
				+ DNP and C.A.	77·11
Fifth instar *Schistocerca gregaria*					
5	18	1	14	Control	72·74
				+ C.A.	63·51
				+ DNP	82·85
				+ DNP and C.A.	85·87
			(b) Mitochondria from thoracic muscle		
Adult *Locusta migratoria*					
6	30	5	17	Control	32·25
				+ C.A.	44·75
				+ C.C.	31·53
				+ protocerebrum	41·32
7	30	6	30	Control	36·58
				+ C.A.	40·43
				+ C.C.	26·25
				+ protocerebrum	35·02

DNP = 2 : 4–dinitrophenol. C.A. = corpus allatum. C.C. = corpus cardiacum.

contrary to the usual reports of the action of this drug at the concentrations used; however, SLATER and LEWIS (1954) found occasional inhibition in young blowflies.

The effect of insect hormones on the oxygen consumption of the mitochondrial/succinate complex was found to be as follows:

The corpora allata hormone added by crushing a pair of corpora allata in the incubation mixture was found to stimulate the oxygen consumption (Fig. 2). That respiration in the intact insect (THOMSEN, 1949; DE WILDE and STEGWEE,

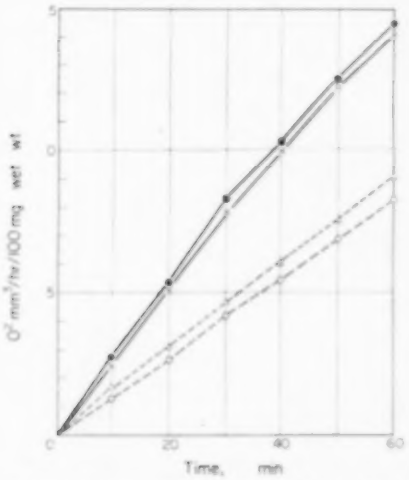

FIG. 1. Effect of DNP upon oxygen consumption of the mitochondria from the fat body of *Locusta migratoria* L. adult. ● —— ●, × —— ×, controls; ○ - - - ○, △ - - - △, + DNP.

1958) and in tissue homogenates (DE WILDE and STEGWEE, 1958) is stimulated by the corpora allata is well known. Its action on mitochondria is reported here for the first time. It is interesting to note that the percentage stimulation found, 8–20 per cent, is comparable to the 19 per cent found by THOMSEN (1949) when three corpora allata were implanted into normal females of *Calliphora*.

The effect of the corpora cardiaca in the two experiments performed was to inhibit respiration (Fig. 2). In one experiment the effect was very slight, in the other the inhibition was quite pronounced.

The effect of the protocerebrum was difficult to estimate. Unlike the other two glands the amount of tissue added to the system was considerable and its bulk might well have been sufficient to give an apparent stimulation. This, perhaps, is what has happened in one case; in the other, marked inhibition occurred although a similar amount of tissue was added in both cases.

Unfortunately there was only time to perform one experiment on individuals from the fifth instar of *Schistocerca gregaria* (Fig. 4). This was one of the last experiments performed and its results are opposite to those found for *Locusta migratoria*. The effect of the corpora allata was to depress the oxygen consumption of the mitochondria, whilst that of the DNP was to stimulate it.

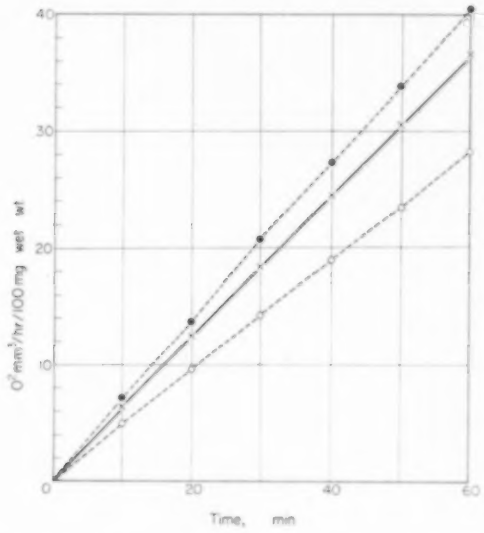

FIG. 2. The effect of the addition of the corpus allatum and the corpus cardiacum to mitochondria prepared from the fat body of adult *Locusta migratoria*. ● - - - ● corpus allatum; × —— × control; ○ - - - ○ corpus cardiacum.

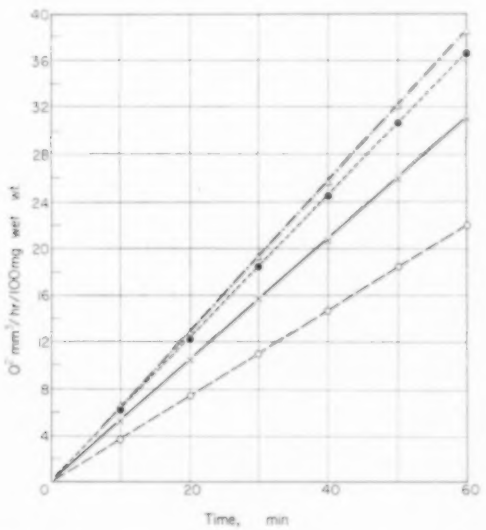

FIG. 3. The effect of the addition of: corpus allatum ● - - - ●; corpus allatum plus DNP △ - - - △; DNP ○ - - - ○; to mitochondria prepared from the fat body of adult *Locusta migratoria*. × —— × control.

The opposite effect of the corpora allata and of the DNP is well marked particularly in the case of the fifth instar where their actions compared with the adult are reversed. The effect of both substances present in the same system was studied. Two experiments were performed in which the action of DNP and corpora allata hormone alone and together were compared with a control on the

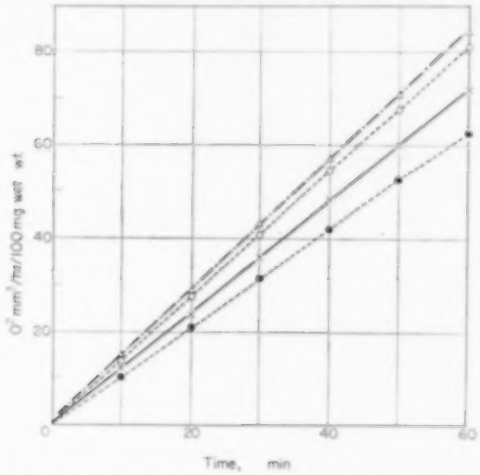

FIG. 4. The effect of the addition of: corpus allatum ● - - - ●; corpus allatum plus DNP △ - - - △; DNP ○ - - - ○; to mitochondria prepared from the fat body of late fifth instar *Schistocerca gregaria*. × —— × control.

same preparation of mitochondria. The results are shown in Figs. 3 and 4. In *Locusta migratoria* the DNP alone inhibited respiration and the corpora allata hormone alone stimulated it; both together resulted in stimulation which was a little greater than that produced by the corpora allata alone. In spite of their opposite effects alone in late fifth instar *Schistocerca gregaria*, both together also resulted in stimulation in this insect (Fig. 4).

DISCUSSION

In the preparation of particles from the fat body and thoracic muscles the procedures of BELLAMY (1958) for *Schistocerca gregaria* were carefully followed. He found, as did REES (1954) for *Locusta migratoria*, that these particles were similar to those isolated from the brain and liver of the rat which had been shown to have properties of mitochondria. Our main modification of BELLAMY'S technique was to omit the final washing, which probably means that our preparations were less pure than his, as indicated by our higher yields. However, when we applied our technique to *Schistocerca gregaria* our percentage wet weight yields were similar to those obtained by him. Our preparations from *Locusta migratoria* under the light microscope showed no nucleoli or cellular debris, so that it is possible that there is a real difference in the mitochondrial content of the two animals.

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The respiration of tissues was studied in a succinate substrate since succinic dehydrogenase is confined to the mitochondrion (SCHNEIDER, 1959); the oxidation of succinate is less influenced by ATP turnover than other steps in the Krebs' cycle and will proceed in the absence of a hydrogen acceptor (EMMELOT *et al.*, 1959). Further, the step from succinate to fumarate involves the acceptance of two hydrogen ions by a flavoprotein, a step which occurs in the metabolism of several other substances and therefore a possible candidate for the site of action of a respiratory controller (KREBS and KORNBERG, 1957).

In all cases in *Locusta migratoria* the effect of DNP was to depress the oxygen consumption in the simple mitochondrial/succinate system used. This is contrary to the observations of most workers although SLATER and LEWIS (1954) do record up to a 16 per cent inhibition with 10^{-4} M DNP in sarcosomes of the blowfly. They also state that they could not obtain stimulation unless Versene was added to their preparations. As Versene is known to increase ATP-ase activity and to inhibit succinic dehydrogenase it was not used in these studies.

The effect of the corpus allatum hormone in stimulating respiration is in line with the many observations that its addition to homogenates (DE WILDE and STEGWEE, 1958) and its injection into intact animals has a similar effect, and that its removal (THOMSEN, 1949) causes a fall in oxygen consumption in blowflies. Here it is shown that it acts directly on the mitochondrion and that its percentage increase in activity is the same as that found in the intact animal, suggesting that this is perhaps its sole site of activity.

Of particular interest is the opposite action of the corpora allata hormone to that of DNP when each is present alone in the system. When they are present together, the inhibitory effect of DNP disappears and the stimulation obtained is a little greater than that of the corpora allata acting alone. The known action of DNP may provide a clue to the action of the corpora allata hormone on the mitochondrion. DNP is one of many agents known to cause oxidative phosphorylation. In many cases this is accomplished by the activation of an ATP-ase in the mitochondrion (SCHNEIDER, 1959). In addition to this, DNP can increase the rate of oxygen consumption by influencing the hydrolysis of high energy phosphate bonds (SLATER and LEWIS, 1954). DRYSDALE and CONN (1958) also suggest that the hydrolysis of ATP is catalysed by DNP but do not think that this is its sole site of action.

The system under consideration is too complex for the few clues provided here to allow any definite site of action to be suggested. It is worth noting that ATP is one of the high energy sources used in most processes that occur in the cell. Its control by a hormone would virtually stop or stimulate the overall activity of the cell. Removal of the corpora allata has resulted in the failure to synthesize protein in *Dixippus* (L'HELIAS, 1953) and has resulted in an increase in fat in *Melanoplus* (PFEIFFER, 1945) and *Periplaneta* (BODENSTEIN, 1953). ATP is necessary for peptide synthesis, and the formation of ATP is accomplished in part by the reduction of ketoglutaric acid to succinic acid in the Krebs' cycle. In the metabolism of fat, degradation products are fed into the Krebs' cycle at the acetyl co-enzyme A level.

These effects of the corpora allata hormone could be explained by assuming its site of action lies with the Krebs' cycle or on the cytochrome transport system.

The inhibition of oxygen consumption in the late fifth instar of *Schistocerca gregaria* and its stimulation by DNP is opposite to the action of these substances in adult *Locusta migratoria*. It should be noted that at this time the corpora allata hormone is present in very low concentration in the locusts. However, one experiment does not offer sufficient evidence to justify any further discussion.

The results of the other experiments call for little discussion. In both experiments the corpus cardiacum inhibited respiration but only slightly so in one case. No conclusions can be drawn about the effect of the protocerebrum, the technique used is obviously not suitable when large amounts of tissue have to be added as carriers of the hormones used, but is quite adequate when as in the case of the corpora allata a very small amount of tissue is all that has to be added.

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SUMMARY

(1) Measurements have been made of the oxygen consumption of mitochondria prepared from the fat body and thoracic muscles of adult *Locusta migratoria* and fifth instar *Schistocerca gregaria*. The effects of various substances upon respiration of mitochondria have been measured.

(2) The addition of 2 : 4–dinitrophenol (DNP) to mitochondria prepared from adult locusts was to depress their oxygen consumption, while in the fifth instar the effect was to stimulate respiration.

(3) The addition of corpora allata to mitochondria prepared from adult locusts resulted in a stimulation of oxygen consumption while in the fifth instar its addition depressed respiration.

(4) The addition of both the corpora allata and the DNP together resulted in stimulation of oxygen consumption both in adult and fifth instar locusts. The oxygen consumption in both cases being a little higher than that caused by either substance working alone.

(5) The addition of the corpora cardiacum to preparations of mitochondria from adult locusts resulted in a depression of oxygen consumption. This was slight in one case and considerable in another.

(6) The possibility of the corpora allata controlling metabolism by its action on the mitochondrion is discussed, as is also the possibility of the known action of DNP offering a clue to the site of action of the corpora allata hormone.

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ESTIMATION OF THE COMPOSITION OF THE CUTICULAR WAX OF THE MORMON CRICKET, *ANABRUS SIMPLEX* HALD.*

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Abstract—The lipid solvent extractable material from the cuticle of *Anabrus simplex* Hald. (Mormon cricket) has been examined in an effort to elucidate its chemical composition. Hydrocarbons, free fatty acids, esters, free cholesterol, and acidic resins are present in measurable amounts. Infra-red spectrophotometry, column chromatography, and gas chromatography have been utilized for the separation and characterization of these various components.

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INTRODUCTION

THE cuticular 'wax' of insects has been recognized as an important factor in the mechanism for the control of their water balance. Evidence of this has been presented in a review by WIGGLESWORTH (1957). Numerous papers establish that the mechanism for this control lies within the lipid-like layer. However, only a few investigators have considered the chemical composition of the 'wax' itself. Comparison of 'wax' composition as presented in papers by HACKMAN (1951), BEAMENT (1955), GILBY and ALEXANDER (1957), and TANG (1939) indicate that considerable variation may be expected among species. The composition of insect wax cannot, therefore, be narrowly defined. Each wax must be studied individually. This paper presents the results of an investigation of the composition of the cuticular wax of the Mormon cricket, *Anabrus simplex* Hald.

MATERIAL AND METHODS

(a) *Preparation of sample*

The crickets were collected from various infestations located in south-central Montana. No selection as to sex or age was made, but the crickets were predominantly in the adult stage. The crickets were returned to the laboratory and killed by subjecting them to chloroform and ammonia vapours in a closed container. The presence of the ammonia in the killing medium appeared to reduce the regurgitation of fluids from the crop. The crickets were then washed by spraying with water, air dried, and frozen. The immediate surface of the frozen crickets was allowed to thaw which permits ready removal of the abdominal sclerites with a minimum of

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adhering extraneous material. Any remaining extraneous material was removed by careful brushing under water. The sclerites obtained in this way were dried and refrozen for later extractions with appropriate solvents.

Extraction trials using chloroform, benzene, constant boiling chloroform–methanol, and 'Skellysolve-B' as solvents in a soxhlet apparatus were equally effective. Subsequent study failed to show any measurable differences in the 'wax' obtained from these several solvent systems. Most of the data was obtained from 'wax' extracted with 'Skellysolve-B' because this system did not emulsify to the extreme degree that the other systems did.

The extracts obtained in this way were washed with water and dried over sodium sulphate. The solvent was removed by evaporation at room temperature using a stream of nitrogen to hasten the evaporation.

(b) *Alumina chromatography*

'Wax' samples were introduced onto the column (16 mm × 30 cm glass, fitted with a 'teflon' stopcock) with a minimum of petroleum ether as solvent. The column was packed with adsorption alumina (Fisher Scientific Co., A-540) in a ratio of about 3 g of alumina for each 0·1 g of sample introduced.

Elution was made by passing approximately 50 ml of each of the following solvents through the column in their respective order: petroleum ether, 1 : 1 petroleum ether–benzene, benzene, 1 : 1 benzene–diethyl ether, diethyl ether, 1 : 1 diethyl ether–acetone, acetone, 1 : 1 acetone–methanol, and methanol.

Fractions averaging about 4 ml each were cut at 10 min intervals with an automatic fraction collector.

(c) *Identification of fractions obtained from column chromatographic separations*

An estimate of the chemical nature of the various fractions was made from their respective infra-red absorption spectra. All spectra, with one exception, were taken in the 5000–680 cm^{-1} region from films cast onto salt plates (either directly or from a suitable solvent). The exception involved a fraction that did not form a suitable film and was run as a solution in carbon disulphide. The interpretations of spectra were made by comparison with the spectra of known compounds and/or reference to specific band assignments as found in the literature. Frequent reference was made to a review paper by O'Connor (1956).

(d) *Gas chromatography of methyl esters and hydrocarbons*

Gas chromatography was used in the elucidation of the hydrocarbons and of the fatty acids.

The hydrocarbons were run with a 2½ ft silicone column at 255°C as well as a 5 ft Apiezon column at 250°C.

The fatty acids were converted to their methyl esters, cf. (f), and run at 210°C on a 5 ft 'Craig' polyester column (Wilken's Instrument Co.).

An estimation of the relative amounts of the various fractions, both hydrocarbons and methyl esters, was made by applying the area data to the

empirical formula $(w_i/W) = (A_i \sqrt{M_i})/(\Sigma A_i \sqrt{M_i})$ suggested by EASTMAN (1957). (w_i/W) is the weight fraction of the ith component, and M_i its molecular weight. A_i is the area under the elution curve of ith component.

(e) *Saponification*

Saponifications were made by heating ca. 0·5 g of wax at about 150°C with 5 ml of 5 per cent KOH in ethylene glycol for 2 hr. The products of saponification were obtained from the reaction medium by acidifying with 3 N sulphuric acid and extracting with diethyl ether. The ether extracts were combined, dried with sodium sulphate, and the solvent was then removed. The products of saponification obtained in this way were taken up in 500 ml of *n*-hexane and passed through a 3-stage Kies' counter-current extraction assembly that contained suitable amounts of potassium hydroxide in 30 per cent alcoholic solution. An additional 500 ml of *n*-hexane was then washed through the system. The combined aqueous portions contained the saponifiable moiety and the *n*-hexane that passed through the system contained the non-saponifiable portion. The saponifiables were recovered from the aqueous portion by acidification, extraction with diethyl ether, drying with sodium sulphate, and removal of the solvent. The *n*-hexane solution was dried with sodium sulphate and the solvent was removed to yield the non-saponifiables.

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(f) *Preparation of methyl esters*

An average sample (ca. 0·02 g) of acids was refluxed with 5 ml of 10 per cent sulphuric acid (by wt.) in methanol for 2 hr. The reaction mixture was then diluted to 30 ml with water and extracted with diethyl ether. The ether extracts were back-scrubbed with water, dried with sodium sulphate and then the solvent was removed by evaporation.

DISCUSSION

The extractable cuticular 'wax' accounted for about 4·3 per cent of the air-dried weight of the excised abdominal sclerites. The 'wax' was a light yellow solid at room temperature (ca. 23°C). The substance was soft, had a typical waxy appearance and readily formed films. It gradually softened above 23°C and became distinctly liquid above 50°C.

The infra-red absorption pattern of this 'wax' is shown in Fig 1. The strong band at 1713 cm^{-1} is characteristic of the -C = 0 stretch in fatty acids. The presence of an acid constituent (or constituents) is further indicated by the general appearance of the spectrum and particularly by the broad hydroxyl band extending across the entire carbon–hydrogen stretch region. Esters are suggested by the bands at 1740 cm^{-1} and in the 1100–1200 cm^{-1} region. Unsaturation is probable as indicated by the shoulder at 3025 cm^{-1}. The bands at 720 cm^{-1} and at 730 cm^{-1} are typical of long-chain compounds in the solid state. These data are explainable by the presence of hydrocarbons, free acids, and esters, all of which were isolated from the wax in the course of this work.

The free acids were removed from the wax by passing a 'Skellysolve-B' solution of the wax through a Kies' assembly containing an alcoholic potassium hydroxide solution as the aqueous phase. Fig. 2 is the infra-red spectrum of the

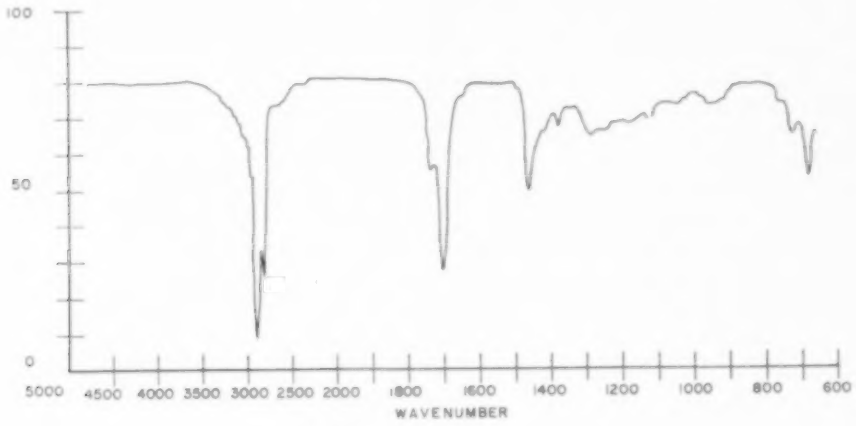

Fig. 1. Cuticular wax.

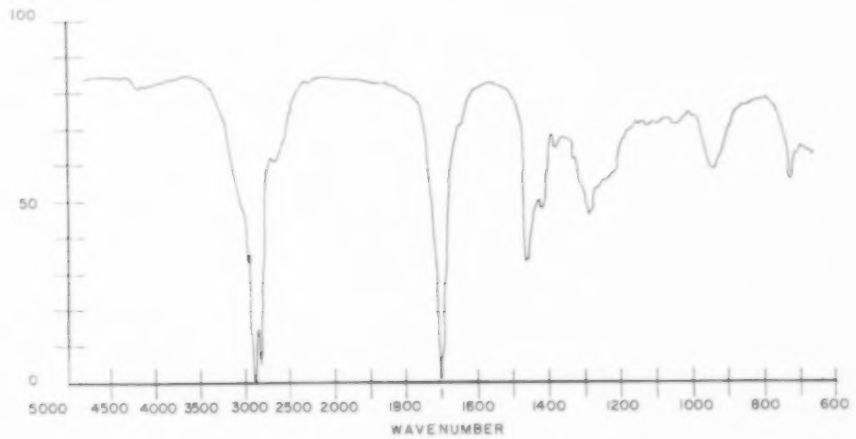

Fig. 2. Free acids of cuticular wax.

acids obtained in this way. These acids represent about 18 per cent of the total weight of cricket wax. Unsaturation is suggested by the carbon hydrogen stretch at 3020 cm^{-1} in this spectrum. These same acids were converted to their methyl esters and another infra-red spectrum was made. The spectrum was typical of a mixture of methyl esters, and in addition suggested some unsaturation. This spectrum gave no indication of free hydroxyl groups (hydroxyl band in the

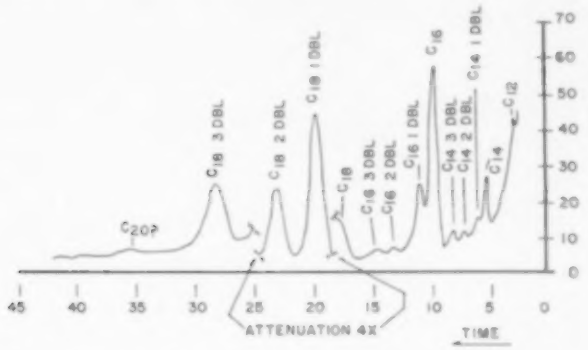

FIG. 3. Gas chromatography of methyl esters of free acids: 5 ft polyester column, 210°C.

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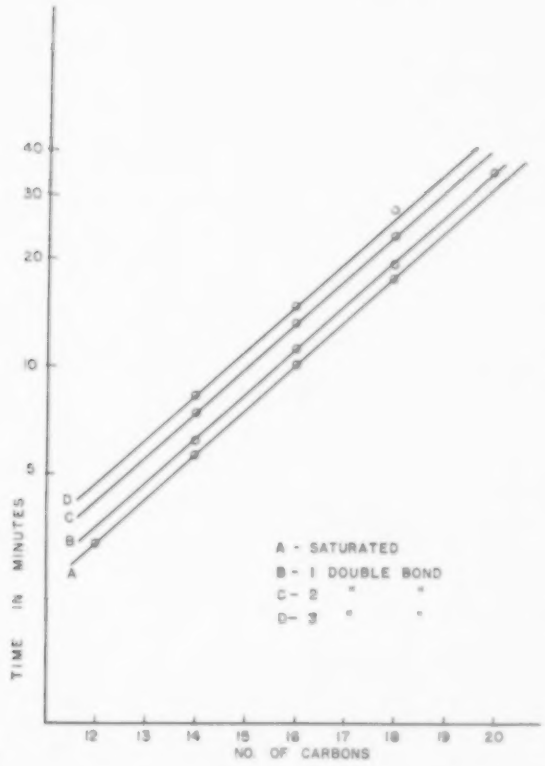

FIG. 4. Gas chromatography retention times of methyl esters of free acids: 5 ft polyester column.

3300 cm^{-1} region was absent). These methyl esters were separated by gas chromatography using a 5 ft 'Craig' column (Wilken's Instrument Co.) operated at 210°C. A logarithmic plot of the retention times obtained from the chromatogram (Fig. 3) is shown in Fig. 4 and indicates the acids shown in Table 1.

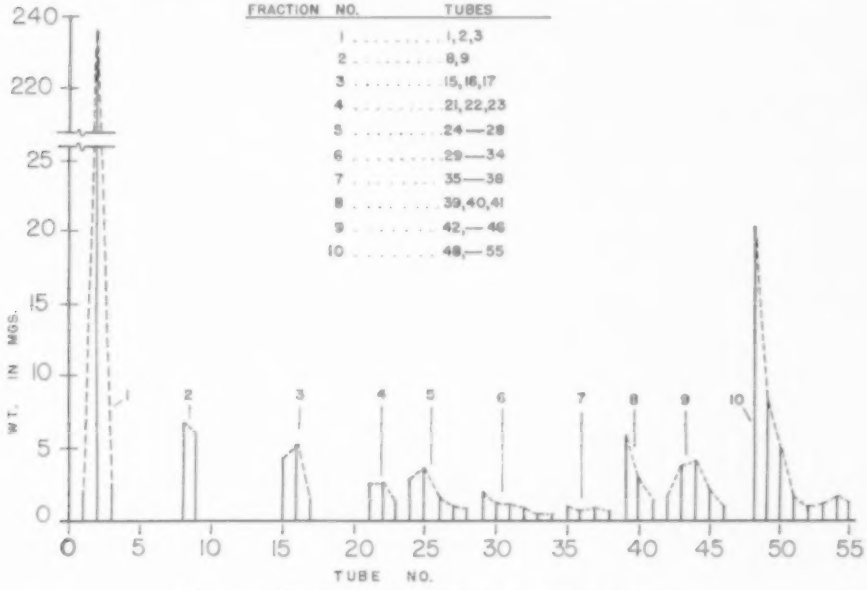

Fig. 5. Alumina chromatography of cuticular wax.

Table 1—Free acids in cricket wax

Type	% of total free acids	% of wax
C_{12}	0·4	0·1
C_{14}	1·7	0·3
C_{14}—1 double bond	0·5	0·1
C_{14}—2 double bonds	0·3	0·1
C_{14}—3 double bonds	0·6	0·1
C_{16}	7·9	1·4
C_{16}—1 double bond	4·0	0·7
C_{16}—2 double bonds	0·3	0·1
C_{16}—3 double bonds	0·7	0·1
C_{18}	2·4	0·4
C_{18}—1 double bond	48·4	8·7
C_{18}—2 double bonds	26·8	4·8
C_{18}—3 double bonds	6·0	1·1
C_{20}	Trace	

The presence of unsaturation in the acids as reported in Table 1 was verified by bromination of some of the methyl ester mixture used for the chromatogram of Fig. 3 and passing this brominated mixture through the same column operated

under identical conditions. In every case where unsaturation is reported, the peak for that particular ester was either markedly diminished or absent. This is the result to be expected when the unsaturated compounds accept the bromine and

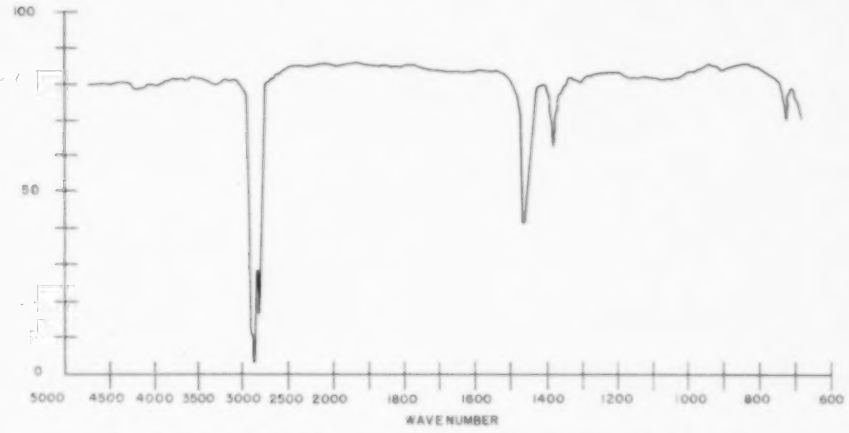

FIG. 6. Fraction No. 1 from alumina chromatography of cuticular wax.

thereby increase their respective retention times to such an extent that they do not appear on the chromatogram.

Fig. 5 shows the results of the chromatographic separation of another sample of cuticular 'wax' on an alumina column. The fifty-five cuts were recombined into

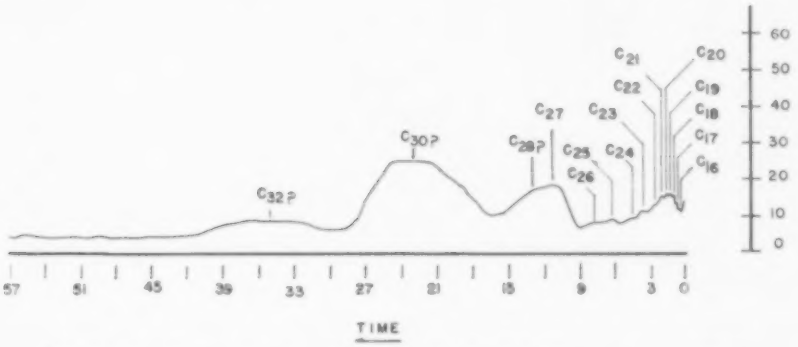

FIG. 7. Gas chromatography of Fraction No. 1: 2 ft silicone column, 255°C.

the ten fractions indicated. The infra-red spectrum of Fraction No. 1 is shown in Fig. 6. It indicates that this white wax, Fraction No. 1, was hydrocarbon in character. The hydrocarbon characteristic was verified by a carbon–hydrogen analysis of this wax; carbon 85·00 per cent, hydrogen 14·96 per cent. Separation of Fraction No. 1 by means of gas chromatography using a 2½ ft silicone column at 255°C gave the chromatogram shown in Fig. 7. This same material was also

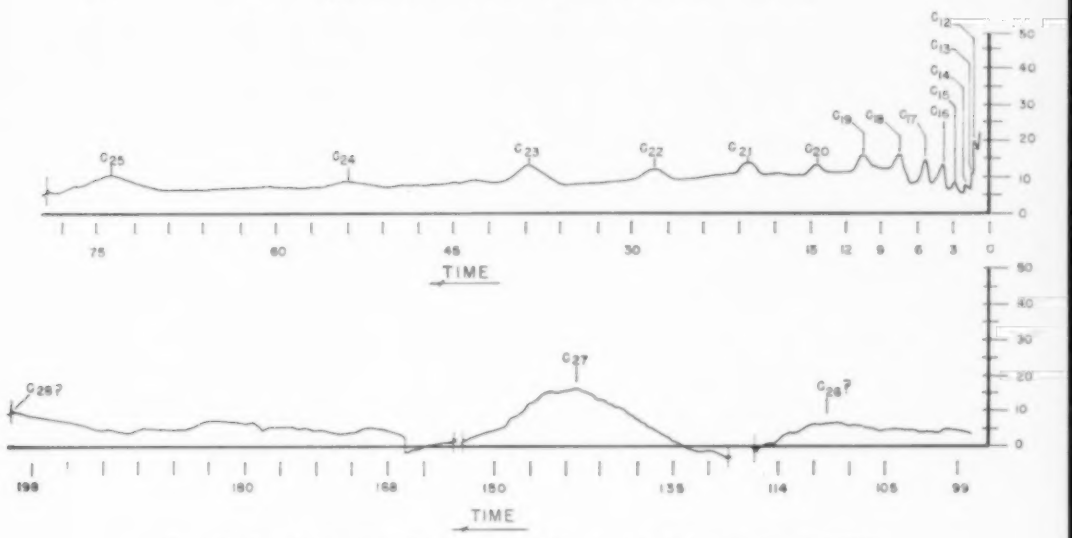

FIG. 8. Gas chromatography of Fraction No. 1: 5 ft Apiezon column, 250°C.

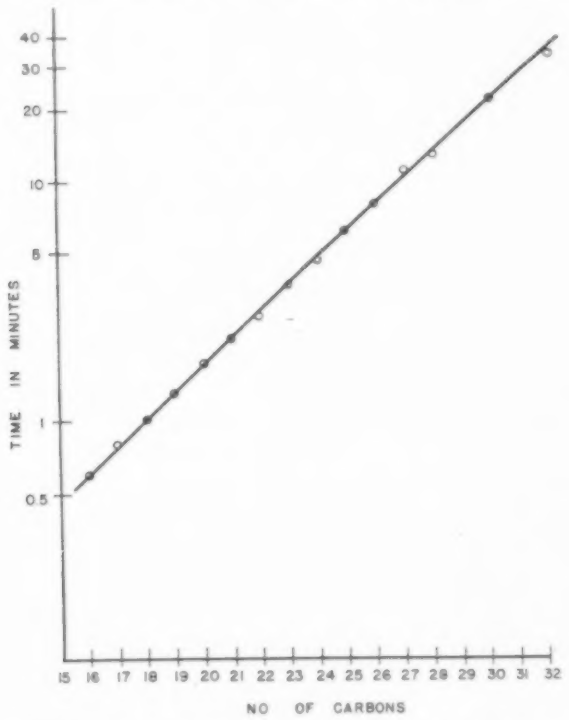

FIG. 9. Gas chromatograph retention times of Fraction No. 1: 2 ft silicone column, 205°C.

separated on a 5 ft 'Apiezon' column at 250°C. The results are shown in Fig. 8. The logarithmic plots of data obtained from these two runs are presented in Figs. 9 and 10 and an estimate of the hydrocarbons and their respective percentages is given in Table 2.

TABLE 2—HYDROCARBONS IN CRICKET WAX

Molecular size	% of total hydrocarbons	% of wax
C_{20} group (C_{19} to C_{25})	12·5	7·2
C_{27} to C_{28}	20·6	11·9
C_{30} to C_{31}	55·3	31·9
C_{32}	11·4	6·6

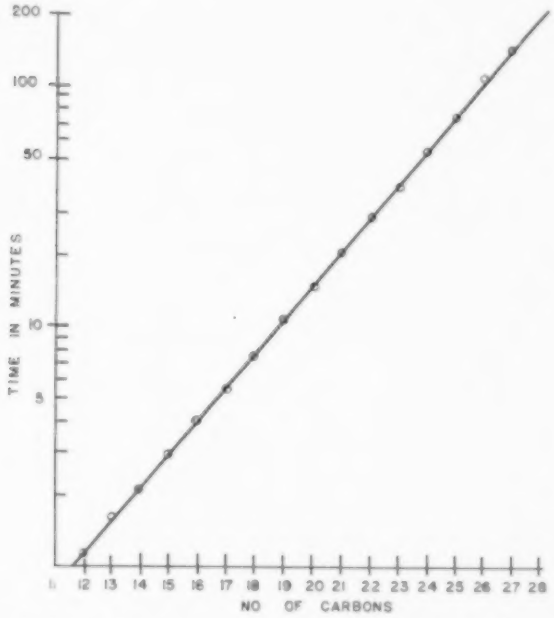

FIG. 10. Gas chromatograph retention times of Fraction No. 1: 5 ft Apiezon column, 250°C.

Fractions 2, 3, and 4 from the alumina chromatography appear from their infra-red spectra to be composed essentially of esters. Differences manifested by their respective spectra (Figs. 11, 12, and 13) are in the extent of unsaturation (highest in Fraction 3) and in the presence of hydroxyl groups (Fraction 4).

Fig. 14 is the infra-red spectrum of a solution of Fraction No. 5 in carbon disulphide. Fraction 5 was rather crystalline in character and did not form films as did the other fractions. It was obviously heterogeneous, having streaks of yellow, waxy material occluded with the white, solid product. Fraction No. 5 is probably cholesterol. This contention is based on comparison of its infra-red

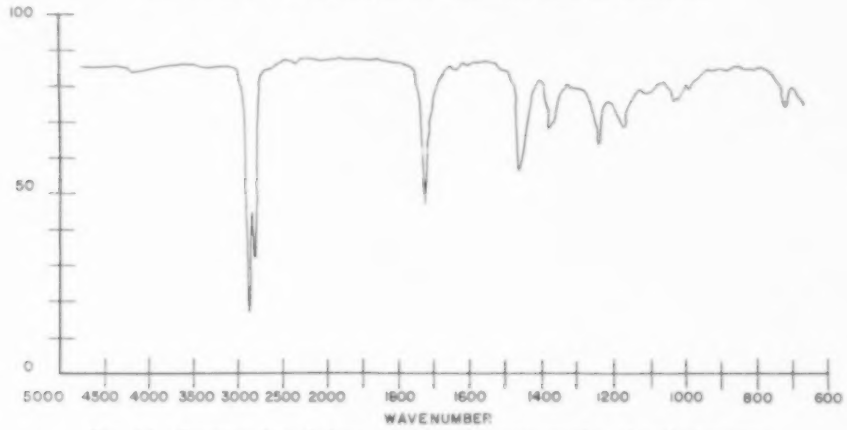

Fig. 11. Fraction No. 2 from alumina chromatography of cuticular wax.

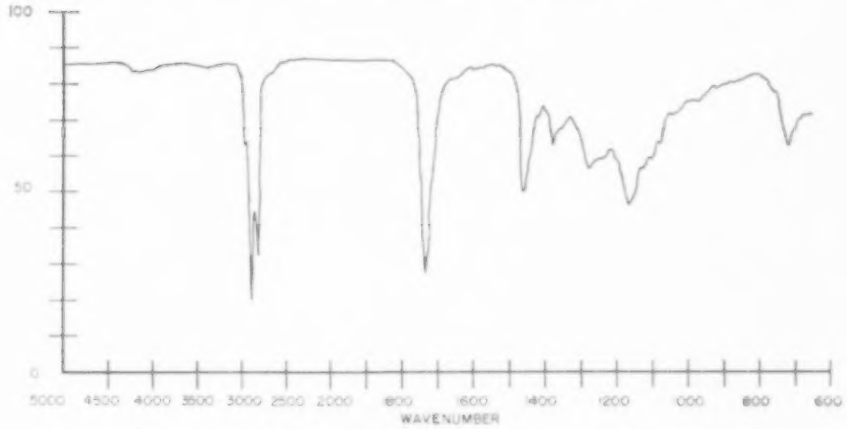

Fig. 12. Fraction No. 3 from alumina chromatography of cuticular wax.

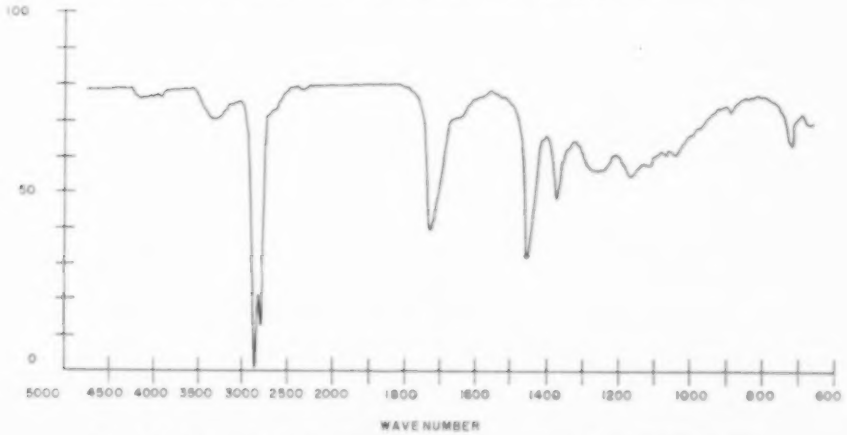

Fig. 13. Fraction No. 4 from alumina chromatography of cuticular wax.

spectrum to that of pure cholesterol (Fig. 15). The spectra of Figs. 14 and 15 are identical if the ester bands at 1736 cm^{-1}, 1170 cm^{-1}, and 720 cm^{-1} are discounted as due to the occluded waxy material of Fraction No. 5.

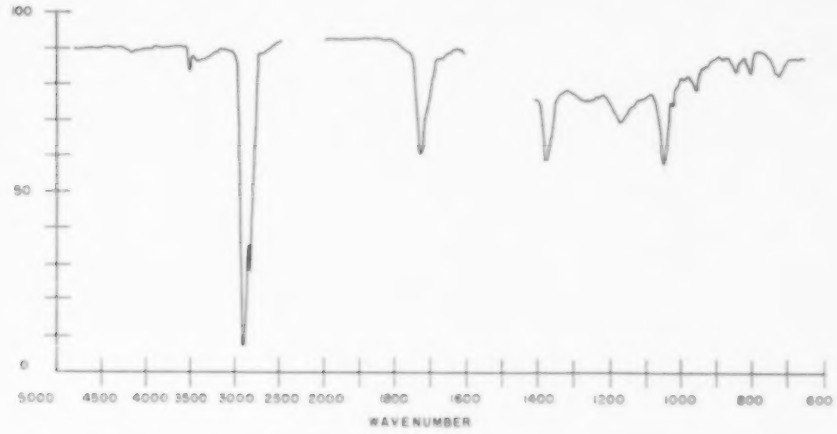

FIG. 14. Fraction No. 5. (in CS_2) from alumina chromatography of cuticular wax.

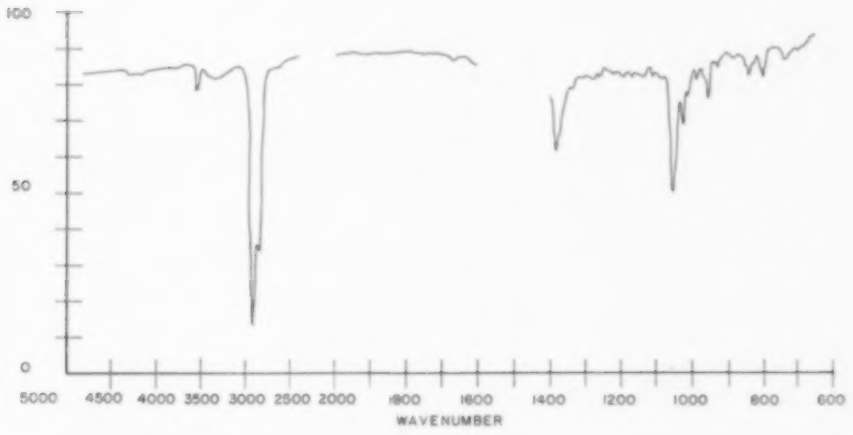

FIG. 15. Cholesterol in CS_2.

The spectra of Fractions 6 and 7 (not shown) were similar to each other and once again suggested esters. The spectra of these cuts indicated both hydroxyl groups and unsaturation whereas the previous ester fraction did not.

Fraction 8 has not been identified at this time. Its infra-red pattern is shown in Fig. 16.

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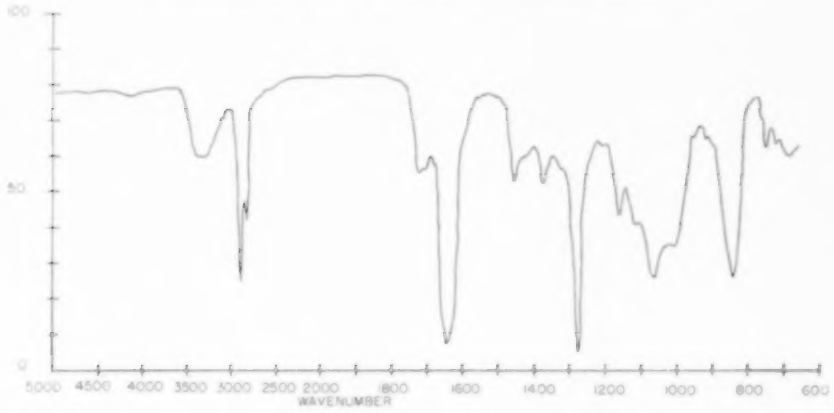

FIG. 16. Fraction No. 8 from alumina chromatography of cuticular wax.

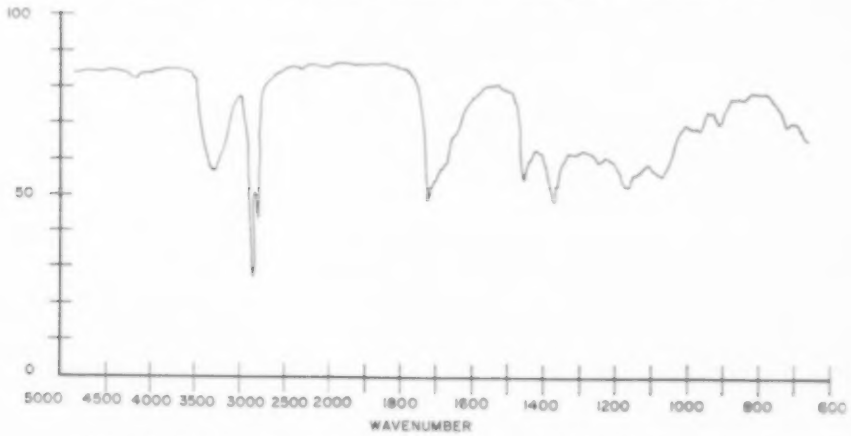

FIG. 17. Fraction No. 10 from alumina chromatography of cuticular wax.

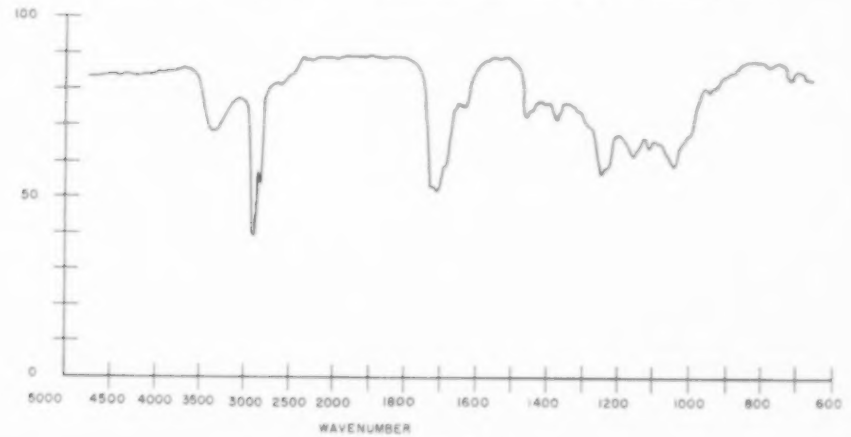

FIG. 18. Shellac.

The spectra of Fractions 9 and 10 were nearly identical. Comparison of the infra-red spectrum of Fraction 10 (Fig. 17) with that of commercial shellac (Fig. 18) suggested a similarity of materials. The physical characteristics of this material are likewise similar to those for shellac. That is, it will form a hard film; is soluble in methanol and warm basic solutions and is insoluble in *n*-hexane and non-polar solvents.

TABLE 3—SUMMARY OF THE COMPOSITION OF MORMON CRICKET WAX

Chemical class	% of wax	Individual component	% of class	% of wax
Hydrocarbons	48–58	C_{12}, C_{13}, C_{14}, C_{15}, C_{16} C_{17}, C_{18}, C_{19}, C_{20}, C_{21} C_{22}, C_{23}, C_{24}, C_{25}, C_{26}	12·5 Equally distributed	6–7
		C_{27}–C_{28}	20·6	10–12
		C_{29}–C_{31}	55·3	27–32
		C_{32} ?	11·4	5–7
Free acids	15–18	C_{12}	0·4	1
		C_{14}	1·7	0·4
		C_{14}—1 double bond	0·5	0·1
		C_{14}—2 double bonds	0·3	0·1
		C_{14}—3 double bonds	0·6	0·1
		C_{16}	7·9	1–1·4
		C_{16}—1 double bond	4·0	0·6
		C_{16}—2 double bonds	0·3	0·1
		C_{16}—3 double bonds	0·7	0·1
		C_{18}	2·4	0·4
		C_{18}—1 double bond	48·4	7–9
		C_{18}—2 double bonds	26·8	4–5
		C_{18}—3 double bonds	6·0	1
		C_{20}	Trace	Trace
Esters	9–11	Saturated	34	3·2–3·9
		Saturated and unsaturated	28	2·7–3·3
		Saturated and/or hydroxy	15	1·4–1·7
		Unsaturated and/or hydroxy	23	2·2–2·7
Free alcohols	2–3	Cholesterol		2–3
Polymers	12–14	Acidic resins of varying molecular size		12–14
Unidentified	2–4			2–4

Recovery of material from the alumina column was incomplete as would be expected with the known presence of free acids which would be difficult to elute. The amount of material left on the column was comparable with the amount of free fatty acids isolated by counter-current extraction.

The amount of material obtained in the previously described individual ester fractions was not sufficient for further elucidation of their structure. However, gas chromatography of the methyl esters of acids obtained from the saponification

of the whole wax did not exhibit any acids differing in type from those shown to be present in the free acids. The percentage of unsaturated acids found in the saponification mixture was slightly higher than that from the mixture of free acids. The esters must, therefore, be somewhat richer in the unsaturated forms.

SUMMARY

An estimate of the composition of the cuticular wax of *Anabrus simplex* Hald. has been made. The investigational nature of our work did not always provide the basis for precise quantitative measurements. Data have been obtained by use of alumina chromatography, gas chromatography, and infra-red spectrophotometry for separation and identification of the individual components. Table 3 is a summary of the probable composition of the wax.

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THE EFFECTS OF DIAPAUSE, DEVELOPMENT, AND INJURY ON THE INCORPORATION OF RADIOACTIVE GLYCINE INTO THE BLOOD PROTEINS OF THE *CECROPIA* SILKWORM*

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Abstract—Glycine-1-^{14}C, injected into the haemocoele of diapausing pupae and developing adults of the *Cecropia* silkworm, is incorporated into the proteins of the blood. The rate of incorporation parallels previously described rates of oxygen consumption (SCHNEIDERMAN and WILLIAMS, 1953) under a variety of conditions. Amino-acid incorporation into blood proteins is thus representative of the endergonic processes occurring in the insect.

The incorporation of amino acids can be added to the list of metabolic processes which proceed at depressed rates during diapause, but which accelerate temporarily in response to injury. The pro-thoracic gland hormone, in causing the termination of diapause in *Cecropia*, evokes metabolic changes which are as yet indistinguishable from those elicited by injury.

The accelerated respiration following injury is probably due primarily to a general acceleration of energy-requiring maintenance processes which are normally suppressed during diapause, rather than to energy invested in the morphogenetic work of wound repair.

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INTRODUCTION

THE development of the *Cecropia* silkworm is interrupted by a period of diapause which is terminated under normal circumstances by the action of an endocrine system including the brain and pro-thoracic glands. The resumption of development can also be evoked by injections of suitable amounts of ecdysone (WILLIAMS, 1954), an isolated hormone which possesses the activity of the pro-thoracic glands (KARLSON, 1956). It is reasonable to presume that the function of this hormone in stimulating the resumption of development is to activate physiological processes which are necessary for development, but which are irrelevant to the maintenance of the diapausing pupa. According to this point of view, an analysis of the biochemical basis of diapause resolves itself into a search for metabolic events which cannot occur in the absence of the pro-thoracic gland hormone.

Among the processes which would be expected to change in character at the termination of diapause is the synthesis of proteins. In the present study we have compared the capacity of diapausing pupae and developing adults to incorporate

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amino acids into proteins. In a preliminary report on this study (TELFER and WILLIAMS, 1955) we noted that diapausing pupae of the *Cecropia* silkworm are able to incorporate ^{14}C glycine into the proteins of the blood, and that the rate of this incorporation is accelerated by prior injury to the pupal integument. This fact complicated our initial objective of determining whether uninjured pupae normally incorporate amino acids, since the injection itself constitutes an injury which we are unable to avoid. Our present purpose is to consider more fully how diapause, development, and integumentary injury influence the kinetics of amino-acid incorporation into the proteins of the blood.

METHODS

Experimental animals

Cecropia silkworms were reared in the field and harvested immediately after they had spun their cocoons. The cocoons were stored for approximately 1½ months at 25°C and then were divided into two groups. One group was maintained at 25°C where the pupal diapause persists for at least 5 months. Prior to the fifth month the brains of the 'unchilled pupae' were excized in order to assure that these animals remained in diapause (WILLIAMS, 1946). The second group of cocoons was stored at 6°C, a treatment which activates the endocrine mechanism for the termination of diapause (WILLIAMS, 1956). For experiments requiring developed animals, groups of 'previously chilled pupae' were removed from their cocoons and placed at 25°C, at which temperature they resumed development within a few days. The second day of adult development is distinguished by the retraction of the leg epithelium from the overlying pupal cuticle. Except in darkly pigmented individuals, this event can be seen in the intact and uninjured insect when the leg region is moistened with 70 per cent alcohol and examined under the dissecting microscope. Later stages in adult development were timed from the second day and checked against the succession of externally visible characters tabulated by SCHNEIDERMAN and WILLIAMS (1953).

Injection of labelled glycine and collection of blood

Each experimental animal was injected with a solution of glycine-1-^{14}C (Tracerlab, $1{\cdot}7 \times 10^{-2}$ mc/mg), which contained, according to our counting procedure, 2×10^6 cpm/ml. The volume of the injected solution was 0·05 ml/g of animal so that each animal received 6 μg or 100,000 cpm of glycine per gramme. This solution was injected from a tuberculin syringe through a 30-gauge stainless steel needle. The needle was inserted through the integument at a point lateral to the midline of the first abdominal tergite. After the injection, the puncture was sealed with melted paraffin. Individuals which showed melanin formation in their blood following the injection were discarded. It was possible to reduce the incidence of this complication by saturating the injected glycine solution with the tyrosinase inhibitor, phenylthiourea.

The injected animals were maintained at 25°C until they were bled. For this purpose a scalpel cut was made lateral to the midline of the thoracic tergum. Approximately 0·2 m. of blood were expressed through the cut into a tube by applying gentle pressure on the abdominal segments. The incision was sealed with melted paraffin and was reopened later if a subsequent sample was desired. The blood was cleared of haemocytes by centrifugation and was then stored at −20°C.

Preparation of the blood proteins for counting

The proteins of the blood were prepared for counting according to the procedures described by SIEKEVITZ (1952). Between 0·1 and 0·2 ml of the cell-free blood were pipetted into a glass homogenizer made from a conical centrifuge tube. Distilled water (3 ml)

followed by 3 ml of 10 per cent trichloroacetic acid were then added. The blood proteins precipitated in this manner were washed twice with 5 per cent trichloroacetic acid; they were then extracted with a 1 : 2 : 2 mixture of chloroform, ethyl alcohol, and ethyl ether in order to remove associated lipids. The precipitate was finally washed with acetone and dried for several hours at 45°C. Acetone was again added and the hardened precipitate was ground to a fine powder and plated by suction on to a weighed disk of filter paper. The plated sample was then dried, counted, and weighed.

The washing procedure effectively eliminated free amino acids from the protein precipitate. Thus when labelled glycine was added *in vitro* to a sample of normal *Cecropia* blood, no radioactivity beyond the normal background was detectable when the proteins had been precipitated and washed in the above manner.

The radioactivity of the plated protein samples was determined with a gasflow counter, appropriate corrections being made for self-absorption. The standard deviation of each measurement did not exceed 3 per cent of the total count.

It was necessary to determine the chemical nature of the radioactivity which appeared in the blood proteins of injected animals. For this purpose, hydrolysed and unhydrolysed blood proteins were treated with ninhydrin in order to liberate as $^{14}CO_2$ the carboxyl groups of any labelled amino acids which were not involved in peptide linkages. These reactions were performed in evacuated U-tubes as described by VAN SLYKE *et al.* (1941b). The carbon dioxide evolved in the reaction was collected in dilute sodium hydroxide and precipitated as carbonate by the addition of barium chloride. After the excess base had been neutralized, the precipitated barium carbonate was plated on a tared disk of filter paper and counted and weighed according to the procedure described above for the protein samples.

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Carbon monoxide inhibition

The effect of carbon monoxide on the incorporation of amino acids into the blood proteins was studied with the use of the pressure chamber described by SCHNEIDERMAN and FEDER (1954). Immediately after being injected with labelled glycine, the experimental animals were placed in the air-filled chamber and compressed with 4 atm of carbon monoxide, thus establishing a carbon monoxide : oxygen ratio of 20 : 1. After a specified time, the chamber was decompressed over 1 hr interval. Blood samples were immediately collected and studied as described above.

RESULTS

Incorporation during diapause and early adult development

Nineteen diapausing female pupae from which the brains had been removed 2 months previously were injected with radioactive glycine. Blood samples were collected after incubation at 25°C for intervals ranging from 3 hr to 18 days. From one to three successive samples were taken from each animal. The time-course of the appearance of radioactivity in the blood proteins is recorded in Fig. 1. Significant radioactivity was first detected in the blood proteins 12 hr after the injection. The level of activity then increased steadily until a maximum was reached from 3 to 4 days after the injection. This value was then maintained for at least 2 weeks.

The experiment was repeated on fifteen animals which had terminated diapause and attained the second to sixth day of adult development. The results, recorded in Fig. 1, reveal a markedly faster rate of incorporation than that previously encountered in diapausing pupae. Though the final level of radioactivity was the

same in the two cases, this level was reached in less than one day in the developing animals or approximately four times as fast as in diapausing pupae.

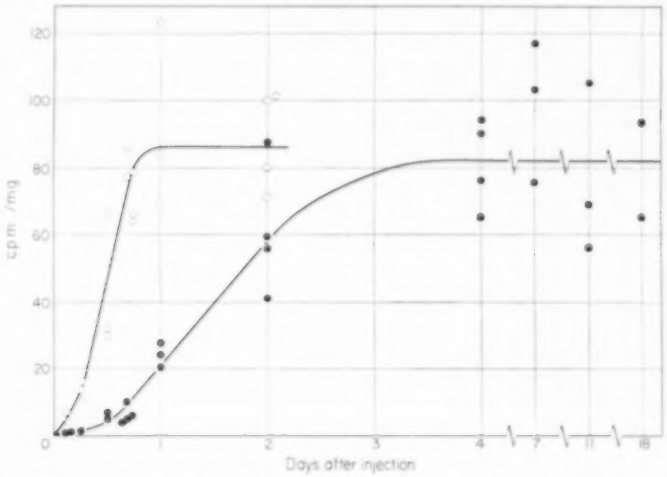

FIG. 1. The radioactivity of the blood proteins following a single injection of labelled glycine into diapausing pupae (closed circles) and developing adults (open circles).

The chemical nature of the ^{14}C *in the blood proteins*

The radioactivity associated with the blood proteins is attributable to amino acids which had probably been incorporated into the peptide structure of the protein itself. The validity of this assertion was demonstrated as follows:

TABLE 1—$^{14}CO_2$ LIBERATED FROM HYDROLYSED AND UNHYDROLYSED BLOOD PROTEINS BY TREATMENT WITH NINHYDRIN

The radioactivity released by ninhydrin is expressed as the percentage of total radioactivity of the protein sample.

Blood protein of diapausing pupae (%)		Blood protein of developing adults (%)	
Unhydrolysed	Hydrolysed	Unhydrolysed	Hydrolysed
1	99	0·8	99
0·5	109	2	116

A pair of diapausing pupae and a pair of developing adults were each injected with a large dose of labelled glycine (300,000 cpm/g live weight). Blood samples were collected from the diapausing pupae after 3 days and from the developing adults after 16 hr.

A sample of the precipitated and washed blood protein from each animal was hydrolysed by boiling for 24 hr in 6 N HCl. When the hydrolysate was treated

with ninhydrin, the evolved carbon dioxide contained as much radioactivity as had been detectable in the original sample of protein (Table 1). Thus, the ^{14}C associated with the protein was entirely in the form of carboxyl groups of amino acids (VAN SLYKE *et al.*, 1941a). A second sample of protein was ground to a fine powder and then treated with ninhydrin without prior hydrolysis. In this manner the carboxyl groups of any amino acids which were not bound to the protein by their amino or carboxyl groups should have been liberated as carbon dioxide. Negligible amounts of labelled carbon dioxide were recovered (Table 1). The labelled amino acid was therefore bound to the protein in a form which made it unavailable for reaction with ninhydrin (SIEKEVITZ, 1952).

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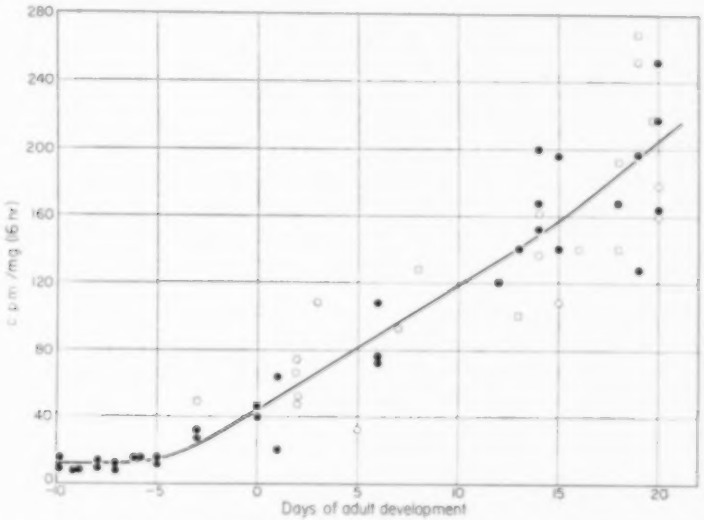

FIG. 2. The radioactivity of the blood proteins 16 hr after a single injection of labelled glycine into insects at various stages of adult development. The closed circles represent females and the open circles represent males. Day '0' represents the initiation of development as indicated by the retraction of the epidermis from the cuticle.

Rate of incorporation during the course of adult development

Thus far, a four-fold difference between the rate of amino acid incorporation during diapause and early adult development has been demonstrated. The stage at which this increase occurs was established by injecting the labelled glycine into diapausing pupae and animals at specific stages of adult development. Blood was collected from each animal 16 hr after it had been injected since, at this time, the radioactivities of the blood proteins of diapausing and developing animals are maximally different.

The results are summarized in Fig. 2. It is evident that the low rate of amino acid incorporation persists until several days prior to the initiation of adult development. The 16-hr level of blood protein radioactivity then begins to increase and

continues to do so throughout adult development. By the time the moth is ready to emerge the 16-hr blood protein radioactivity is 5 times that of the animal at the initiation of adult development and nearly 20 times that of diapausing pupae.

Amino acid incorporation in injured pupae

The slow rate of amino acid incorporation during the pupal diapause parallels the low rate of respiratory metabolism at this time (SCHNEIDERMAN and WILLIAMS, 1953). By means of integumentary injury it is possible to evoke an increase in the metabolism of diapausing pupae to levels which equal or exceed that of animals

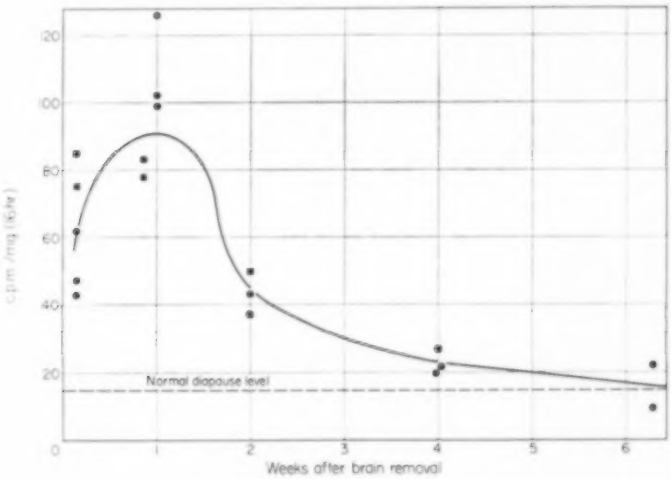

FIG. 3. The radioactivity of the blood proteins 16 hr after a single injection of labelled glycine into diapausing pupae at various times following injury. The injury consisted of the surgical removal of the brain.

at the outset of adult development. The injury response is of special interest because the increased rate of metabolism is not accompanied by any obvious morphogenetic activity, aside from the localized repair of the injury itself.

The effect of injury on amino acid incorporation was investigated in the following manner. Eighteen diapausing female pupae were briefly anaesthetized with carbon dioxide. The cuticle and epidermis were excized from the facial region, the brain was removed, a few crystals of phenylthiourea were added, and the opening was covered with a plastic slip which was sealed in place with melted paraffin. At a specified time following this standardized injury, each individual received a single injection of labelled glycine. Sixteen hours after the injection, a sample of blood was withdrawn. From determinations of the radioactivity of the proteins from these samples, one obtains a plot of the rate of amino acid incorporation as a function of time after injury (Fig. 3).

It is apparent that the injury caused a prompt increase in the rate of incorporation. Indeed, by the second day after injury, the rate of incorporation was considerably higher than in animals on the first day of development. The high rate of incorporation continued for over a week and finally returned to the normal diapausing level after 4 weeks. This result suggests that the injury metabolism reflected in the oxygen consumption is coupled to the synthetic machinery of the diapausing pupa.

Effects of carbon monoxide on amino acid incorporation

The metabolism of diapausing *Cecropia* pupae is resistant to high pressures of carbon monoxide. By contrast, the same treatment blocks the development of post-diapausing animals and reduces their rate of oxygen consumption to the old diapausing rate (SCHNEIDERMAN and WILLIAMS, 1954a, b). In order to examine further the relations between the rate of oxygen consumption and the rate of amino acid incorporation, we investigated the effects of carbon monoxide on glycine incorporation into blood proteins.

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TABLE 2—THE EFFECTS OF CARBON MONOXIDE ON THE APPEARANCE OF RADIOACTIVITY IN THE BLOOD PROTEINS OF DIAPAUSING PUPAE

The pupae were bled 30 hr after an injection of labelled glycine

1 atm air (cpm/mg)	1 atm air 4 atm CO (cpm/mg)
45	31
51	39
20	47
16	20
68	35
	29
Mean 40	Mean 34

$p = 0{\cdot}6$ (t test)

Twelve diapausing pupae were injected with labelled glycine and divided into two groups. Six individuals were immediately exposed to carbon monoxide as described under Methods. The other six were maintained in air. Thirty hours later, blood samples were collected from all twelve animals and assayed for blood protein radioactivity. In the results recorded in Table 2 it is clear that the incorporation of glycine by diapausing pupae was not significantly inhibited by the high pressure of carbon monoxide.

The experiment was repeated with eight animals which had already attained the third to sixth day of adult development. Five were exposed to carbon monoxide

while three were maintained as controls in air. The data summarized in Table 3 indicate a 90 per cent inhibition in the 16-hr level of blood protein radioactivity.

Therefore, it is clear that carbon monoxide affects amino acid incorporation in the same manner as it affects respiration.

TABLE 3—THE EFFECTS OF CARBON MONOXIDE ON THE APPEARANCE OF RADIOACTIVITY IN THE BLOOD PROTEINS OF DEVELOPING ADULTS
The animals were bled 16 hr after an injection of labelled glycine

1 atm air (cpm/mg)	1 atm air 4 atm CO (cpm/mg)
86	8
75	5
79	9
	6
	9
—	—
Mean 80	Mean 7

DISCUSSION

Amino acid incorporation in diapausing pupae

During the pupal diapause, the respiratory rate of the *Cecropia* silkworm is only 2 per cent of that of the mature larva and less than a third of that recorded for animals which have just begun adult development (SCHNEIDERMAN and WILLIAMS, 1953). Despite its low magnitude, the respiratory rate remains finite: the diapausing pupa continues to utilize energy, presumably to maintain its tissues in a viable state. But precisely what are the energy-requiring processes of diapause and how are they modified or added to during the transition from diapause to development?

The observations described here demonstrate that labelled glycine, injected into the haemocoele of diapausing *Cecropia* pupae, can be incorporated into the proteins of the blood. DEMJANOWSKI *et al.* (1952) obtained a similar result with ^{35}S-labelled methionine in diapausing pupae of *Antheraea pernyi*; in addition, they describe the appearance of the amino acid in the proteins of a number of pupal tissues. Finally, BRICTEAUX-GREGOIRE *et al.* (1956) identified glycine-1-^{14}C in the tissue proteins of two diapausing *Sphinx ligustri* pupae after injection with the labelled amino acid. These results implicate at least three processes which may occur during diapause and which presumably require energy: the penetration of cells by amino acids, their incorporation into proteins, and the secretion of proteins into the blood.

Whether the uninjured pupa normally incorporates amino acids into proteins during diapause is a question which will remain unanswered until a means is found for introducing substances into the haemocoele without damaging the integument.

We should note in this connexion that the protein composition of pupal blood does not change appreciably during the months of diapause (CHEFURKA, 1953; TELFER and WILLIAMS, 1953). Thus, if amino acid incorporation occurs during diapause, it is either in equilibrium with the degradation of proteins, or else it results from energy-requiring exchange reactions (GALE and FOLKES, 1953) which do not affect the total amount of protein.

That the processes involved in amino acid incorporation are representative of the endergonic mechanisms of the insect is indicated by the general parallel between the respiratory rates reported by SCHNEIDERMAN and WILLIAMS (1953, 1954a) and the rate of appearance of labelled amino acid in the proteins of the blood. These two measurements of metabolic activity have the following points in common: a low rate during diapause; a relatively high rate following injury and after the termination of diapause; continued acceleration during the later stages of adult development; insensitivity to carbon monoxide during diapause (CO : O_2 ratio of 20 : 1); and inhibition by this level of carbon monoxide during adult development. This correlation implies that the release of energy in the insect is coupled to energy-consuming processes which are exemplified by the incorporation of amino acids into blood proteins.

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The significance of the injury metabolism

The pupa's response to injury appears to signify a general release from the metabolic restraints of diapause—a release which is remarkably similar in its details to that which occurs when development is resumed. In both situations one records an acceleration in the rate of amino acid incorporation, and in the rate of respiration. Cytochrome-*c* appears in the wing epithelium of injured diapausing pupae at a level characteristic of developing animals (SHAPPIRIO, 1958) and, as in the case of the developing animal, the enhanced respiration of the injured diapausing pupa is inhibited by carbon monoxide (HARVEY, 1956; KURLAND and SCHNEIDERMAN, 1958).

Despite these physiological similarities, none of the morphogenetic progress of adult development is visible at any time during the several weeks of injury-stimulated metabolism. The pupa invariably remains in a state of developmental arrest until exposed to the hormone of the pro-thoracic glands. It is necessary to conclude that the pro-thoracic gland hormone has additional effects—as yet undefined—which injury fails to elicit and which are necessary for the resumption of morphogenesis.

Further comparisons between developing and injured animals suggested that the accelerated oxygen consumption following injury is not a measure of the work required to close the wound. For example, during the first 2 weeks of adult development the insect completes numerous steps in the formation of the moth. Included are such processes as the secretion of the moulting fluid, the segmentation of the legs and antennae, the differentiation of scales and hairs on the surface of the epidermis, the beginnings of cuticle formation, the growth and formation of the

genitalia, the differentiation of the germ cells, and numerous other events. But, during a comparable period of stimulated metabolic activity in the injured diapausing pupa, the only detectable morphogenetic accomplishment is the laying down of a simple epidermal layer across the wound (SCHNEIDERMAN and WILLIAMS, 1954b). The comparison demonstrates that the metabolism of injury provides considerably more energy than would be required simply to close the wound. The entire metabolic machinery which will later be utilized to energize the biochemical transformations underlying metamorphosis is activated in synchrony with the lesser task of healing a wound. Our studies of amino acid incorporation indicate that the excess energy may be utilized in processes such as enhanced protein turnover and membrane transport. In effect, the injured animal, in addition to repairing its wound, is stimulated to accelerate its rate of maintenance.

We are led by these considerations to propose the existence in the diapausing pupa of special devices which decrease the work required in order to maintain what GODDARD (1948) has termed the 'thermodynamically improbable structure' of the living organism. Such devices may consist of permeability barriers, the inactivation of hydrolytic enzymes, or other conservative processes akin to those invoked to explain the low metabolic rates of the dormant seeds and spores of plants (GODDARD, 1948). The accelerated metabolism of both injured and developing animals may thus be attributed, at least in part, to the loss of special mechanisms for depressing the cost of maintenance. It is relevant to note that an enhanced metabolism is essential for the resumption of metamorphosis (SCHNEIDERMAN and WILLIAMS, 1954b) and this, according to the view proposed here, indicates that a cell must maintain itself at a suitably fast pace before it is capable of undertaking morphogenesis. In neither injury nor development are there grounds for believing that the accelerated metabolism is a valid measure of the energy required for and invested in morphogenesis itself.

The difference between diapause and development

In summary, we fail to observe a fundamental difference between diapausing and developing animals with regard to their capacity to incorporate amino acids into proteins. The diapausing pupa is apparently capable, not only of incorporating amino acids into proteins, but, in response to injury, of doing so at a rate characteristic of developing animals. Indeed, after injury, it can even effect a reconstitution of certain proteins, such as cytochrome-*c*, without interrupting the dormant condition. Inability to synthesize proteins is, clearly, not the cause of the persistence of diapause.

A more fundamental difference between diapause and development concerns the stability of the mechanisms for protein synthesis and utilization. Development is accompanied by the appearance of new blood proteins and the disappearance of others, by changes in the quantity of most blood proteins, and by the differentiation of mechanisms for the selective utilization of proteins (TELFER and WILLIAMS, 1953; TELFER, 1954, 1960). Changes of this type occur in a sequence which is precisely

correlated with the morphological progress of metamorphosis, and apparently mirror progressive changes in the quality and the quantity of the cellular mechanisms for building up and breaking down specific proteins. While the diapausing pupa appears to contain fully functional machinery for the metabolism of pupal proteins, it is unable to alter this machinery according to genetically prescribed patterns for the completion of its metamorphosis.

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COMPOSITION OF THE OOTHECAE OF THREE ORTHOPTERA

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Abstract—Oothecae of *Periplaneta americana* contained 6·5 per cent calcium and this was the only major cation present. Only 35 per cent of the calcium was present as calcium oxalate and in view of the absence of other anions it is suggested that the remainder may be present as a calcium–protein complex. Oothecae of *Blattella germanica* and *Orthodera ministralis*, although containing much less calcium, gave similar results. Chitin is absent from the oothecae of these three Orthoptera.

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INTRODUCTION

THE ootheca of the cockroach is formed from the secretions of two glands—the left and right colleterial glands. The work of PRYOR (1940), BRUNET and KENT (1955), and KENT and BRUNET (1959) has shown that, in the cockroaches *Periplaneta americana* (L.) and *Blatta orientalis* L., the left gland secretes the structural protein, a polyphenoloxidase and the 4-0-β-glucoside of protocatechuic acid, while the right gland secretes a β-glucosidase. When the secretions interact the β-glucosidase acts on the glucoside liberating protocatechuic acid which is oxidized to a quinone. The quinone is believed to cross-link (i.e. tan) the structural protein chains so forming the hard, dark ootheca. In *B. orientalis* (= *Periplaneta orientalis* (L.) of authors) the ootheca has embedded in its substance crystals which are considered to be calcium oxalate and similar crystals occur in the left colleterial gland. In the ootheca the crystals are reported as being most numerous in the vicinity of the indented dorsal line (for reviews of the literature see ITO, 1924; BRUNET, 1952; CLARK, 1958; and STAY *et al.*, 1960). BRUNET (1952) states, without providing any evidence, that crystals of calcium oxalate occur, mixed with protein, in the lumen of the left colleterial gland of *P. americana*. STAY *et al.* (1960) examined the oothecae from twenty-seven species of cockroaches and observed crystals in eighteen species. In two of these species (*P. americana* and *Blattella germanica*) they identified oxalate in acidic (HCl) extracts of the oothecae. From X-ray diagrams they concluded that the crystals were calcium oxalate dihydrate with the exception of one species in which the monohydrate occurred.

Much less work has been published on the oothecae of praying mantids. PARKER and RUDALL (1955) have shown by X-ray and infra-red studies that the oothecae of praying mantids contain small rectangular crystals of calcium citrate while KATO and KUBOMURA (1956) have reported the identification (histochemically) of calcium carbonate in the egg packets of Japanese mantids.

The results presented here are a study of the oothecae of three Orthoptera—two cockroaches and one mantid. Particular attention has been paid to the calcium content and to establishing the absence of chitin.

EXPERIMENTAL METHODS AND RESULTS

(a) *Periplaneta americana*

Examination under the microscope confirmed the presence of crystals in the left colleterial gland. The crystals were insoluble in water and dilute acetic acid but soluble in dilute hydrochloric acid. An HCl (2N) extract containing the crystals gave positive tests for calcium (flame test and dihydroxytartaric acid osazone test (FEIGL, 1954)) and oxalate (precipitation of calcium oxalate). Similar crystals could be seen distributed throughout the substance of the newly formed ootheca, there being no obvious accumulation of crystals along the indented dorsal line.

Oothecae, from which the young cockroaches had hatched, were cut in half along the dorsal line and the remains of the eggs removed by scraping. The oothecae were soaked overnight in water containing a small amount of synthetic detergent, all traces of adhering material removed by rubbing and repeated washing, washed thoroughly in distilled water and finally air dried. Nitrogen content 10·93 per cent* and tests for sulphur were negative.

The oothecae contained 16·5 per cent ash (550°C) and, when intact half oothecae were ashed, they retained their shape. The ash content of individual oothecae varied from 14·8 to 18·0 per cent. Spectrographic analysis of the ash showed calcium as the major cation (99 per cent, equivalent to 6·5 per cent calcium in the ootheca). Of the trace elements detected, iron, lead, and copper lie in the range 0·001–0·01 per cent; aluminium, magnesium, manganese, titanium, and phosphorus in the range 0·01–0·1 per cent and silicon in the range 0·1–1·0 per cent. To identify the anions, finely ground oothecae were extracted with boiling aqueous sodium carbonate and the usual qualitative tests applied to the extract. Of those anions forming insoluble calcium salts only oxalate could be detected—tests for sulphate, phosphate, tartrate, citrate, fluoride, and arsenate were negative. No carbon dioxide could be detected when sulphuric acid was allowed to react with finely ground oothecae, wetted with water containing synthetic detergent, using the technique devised by CONWAY (1947). Carbonates (and bicarbonates) were therefore assumed to be absent. The amount of oxalate present in the sodium carbonate extract was determined by spot colorimetry (FEIGL, 1956). The oxalic acid was reduced to glyoxalic acid and the colour formed with phenylhydrazine matched with that formed by standard solutions of oxalic acid. The oothecae contained 5·2 per cent oxalate†. Attempts to estimate oxalate after precipitation as calcium oxalate were unsatisfactory. Protein from the aqueous extracts was precipitated along with the calcium oxalate and this protein decolourized acidified potassium permanganate.

* All analytical figures are given on a dry weight basis.

† The values reported for phosphate and oxalate are maximum values.

Finely ground oothecae (0·5 g) were heated with normal aqueous potassium hydroxide (50 ml) at 100°C for 3 days, changing the alkali five times during this period. The residue was washed with water (by centrifugation) until the washings were of neutral pH. The residue, which contained some inorganic material, was hydrolysed by heating at 100°C with 5·7 N HCl (5 ml) for 24 hr. The hydrolysate was evaporated to dryness under reduced pressure, the residue dried *in vacuo* over solid potassium hydroxide and dissolved in 10 per cent *iso*propanol (0·5 ml). This solution was subjected to ionophoresis on paper (Whatman No. 3 filter-paper, acetate buffer pH 5 ionic strength 0·19, potential 20 V/cm for the effective length of 50 cm for 60 min (FOSTER, 1952, 1957). No acidic or basic amino acids or glucosamine were detected with ninhydrin but material reacting with ninhydrin remained at the origin. One- and two-dimensional filter-paper partition chromatography (capillary ascent method of WILLIAMS and KIRBY (1948)) was used to identify this material. The solvent systems and spray reagents described by HACKMAN and LAZARUS (1956) were used since the material proved to be a mixture only of amino acids. An approximate estimate of the amount of each amino acid present was obtained by visual comparison of the intensities of the coloured spots with those given by standard solutions of amino acids. Only the following five amino acids could be detected and the figures given are the concentrations in milligrams per gramme oothecae: leucines (0·60), phenylalanine (0·36), valine (0·86), tyrosine (0·40), and proline (1·06). Heating the oothecal material with alkali for 6 days instead of 3 days decreased the total amount of amino acids but did not eliminate them. Moreover the relative amounts of each remained approximately the same.

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(b) *Blattella germanica* (L.)

The oothecae produced by *B. germanica* are less heavily tanned, are thinner and less brittle than those produced by *P. americana*. Very few crystals could be seen in the substance of the oothecae by examination under the microscope and the few crystals that were seen were confined to the indented dorsal edge.

Oothecae from *B. germanica* were subjected to the same treatment and examination as those from *P. americana*. The oothecae contained 14·5 per cent nitrogen, 1·7 per cent ash, 0·41 per cent calcium, 0·06 per cent phosphorus, and 0·25 per cent oxalate. Tests for sulphate, citrate, tartate, and carbonate were negative. No glucosamine or amino acids could be detected in the acid hydrolysate obtained from the residue left after heating the finely ground oothecal material with aqueous alkali.

(c) *Orthodera ministralis* (F.)

Oothecae from which young praying mantids had hatched were cut in half and washed. The oothecae contained 15·1 per cent nitrogen, 0·33 per cent calcium, 0·1 per cent oxalate, 0·05 per cent phosphorus, and 2·0 per cent ash. Spectrographic analysis of the ash showed the presence of calcium, aluminium, silicon, and magnesium as major elements (each probably exceeding 5 per cent), manganese,

phosphorus, and titanium in the range 0·1–1·0 per cent and boron, lead, iron, sodium, and copper in the range 0·01–0·1 per cent. Tests for sulphur, citrate, tartrate, and carbonate were negative. No glucosamine could be detected in the acid hydrolysate obtained from the residue left after heating oothecae with aqueous alkali.

DISCUSSION

The failure to detect glucosamine in acidic hydrolysates of the residues left after heating the oothecae with aqueous alkali confirms the already reported absence of chitin in the oothecae of *P. americana* and *B. germanica*. Previously workers have relied on the failure to get a positive chitosan test. It has now been established that chitin does not occur in the ootheca of the praying mantid *O. ministralis*. The presence of amino acids in the hydrolysate from *P. americana* oothecal residue is interesting, more particularly since only five amino acids are present. These amino acids must represent a peptide or protein fraction which is extremely resistant to alkaline hydrolysis, but the reason for such resistance is not known. The type of bonding responsible for the insolubility of this fraction may be important in the 'tanning' of the structural protein to form the hard ootheca.

So numerous are crystals of calcium oxalate in the left colleterial gland that it would appear that they play an active part in the formation of the ootheca or in its function. The high calcium content of *P. americana* oothecae (6·5 per cent) serves to support this suggestion. BRUNET (1952) suggested that calcium oxalate is probably present with the protein so that when the diphenolic substance (protocatechuic acid) is formed the pH is at an optimum for enzymic and/or spontaneous oxidation of the phenol. However, if this is true, the amount of calcium oxalate present would appear to be greatly in excess of that required. BORDAS (1908, 1909) attributed a structural function to the crystals, similar to that of the inert filler in concrete. STAY *et al.* (1960) also suggest a structural and/or protective function for the crystals. The function of the crystals is still unknown but an important fact which emerges from the results reported above is the excess of calcium over the anions present which could form insoluble calcium salts (water soluble salts would have been lost in the process used for cleaning the oothecae). In oothecae of *P. americana* 65 per cent of the calcium present has not been accounted for whilst for *B. germanica* the figure is 44 per cent. In the mantid ootheca 77 per cent of the calcium has not been accounted for. The fact that the oothecae of *P. americana* retained their shape after ashing indicates that calcium is distributed throughout the substance of the oothecae.

The manner in which this excess calcium is bound is not known but it could be present in association with the structural protein, so stabilizing it prior to tanning. The ootheca, unlike insect cuticles, contain no chitin and a calcium–protein complex may serve a structural purpose similar to that of chitin. It is known that many proteins readily combine with calcium and the complexes so formed have been the subject of much investigation. The linkages which are formed are so stable that the calcium is not removed either by dialysis or by electrophoresis.

Such protein complexes are less soluble and less readily denatured than the parent protein. Calcium does play an active part in some biological reactions. It may do so in the cockroach ootheca although the large amount of calcium (other than oxalate) present in *P. americana* makes this unlikely (GREENBERG, 1944; GURD and WILCOX, 1956). More recently SHASHOUA and KWART (1959) have published a structure for the mucus from the large marine snail, *Busycon canaliculatum*, in which the protein is linked to the carbohydrate through calcium ions.

The work described here was completed before the publication of the paper by STAY *et al.* (1960). The results reported by them for the oxalate content of the oothecae of *P. americana* and *B. germanica* are in general agreement with the results reported by us. The oothecae of *P. americana* are reported as containing 13–15 per cent calcium oxalate dihydrate (i.e. 7·0–8·0 per cent oxalate) and the oothecae of *B. germanica* as containing 0·3 and 0·4 per cent calcium oxalate dihydrate (i.e. 0·16 and 0·21 per cent oxalate). Oxalate was estimated by precipitation as calcium oxalate, solution in sulphuric acid, and titration with potassium permanganate. Their figure for *P. americana* is higher than that recorded by us (5·2 per cent). This difference could be due to biological differences between the cultures although contamination with protein may be a contributing factor when oxalate is estimated by titration with permanganate.

The results reported for the oothecae of *O. ministralis* do not support the work of PARKER and RUDALL (1955) or KATO and KUBOMURA (1956). No citrate or carbonate could be detected and the composition of the oothecae resembles closely that of the cockroaches.

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THE INFLUENCE OF THE QUEEN ON BROOD REARING IN ANTS OF THE GENUS *MYRMICA*

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Abstract—The queen affects larval growth in *Myrmica scabrinodis* and *M. rubra* in such a way as to cause an earlier start, an improved survival, a smaller pupal size, and a diminished production of gynes. The first three effects apply to males as well as females. The magnitude of the size reduction does not regress on larval number if workers are constant but it does rise to a limit if workers are increased and larvae constant. Detailed studies of larval growth and the growth of wing buds in the final instar show that suppression occurs throughout ontogeny and that there are no accelerations of development other than those that normally occur in worker production. This applies to both sexes. Queens vary a good deal in their potency but agree in showing an increase during cultivation, an expression perhaps of a normal seasonal increase; there is no quantitative relation between effect and queen number. Workers taken from an active queen do not carry the effect.

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INTRODUCTION

THE subject of queen control in social Hymenoptera was reviewed by BRIAN (1958) and preliminary data were then given illustrating the influence of the queen on brood rearing in *Myrmica scabrinodis* Nylander. These indicated that larvae reared in groups containing a queen began to grow earlier and continued to grow well, but changed into prepupae sooner and in the end yielded smaller pupae than those reared in groups without a queen. The queen appeared first to concentrate the workers on the larvae and to increase their work rate (a sort of catalysis of larva/worker interaction) and later to cause some modification in larval physiology that brought about an early metamorphosis. The initial catalysis strongly suggested that the simple explanation of size reduction as being due to competition between queen and larvae for food was unlikely. In this paper these results are confirmed and amplified and extended to the species *M. rubra* L.; the effects of varying the numbers of larvae and workers are described, details of larval growth are given, and queen variability and number are considered.

METHOD

All ant groups have been cultured at 23°C and 100 per cent relative humidity with ample food in standard artificial nests. In the experiments to be described last instar larvae after hibernation have been cultured by workers with or without a queen or queens. Both the workers and queens have been prepared by 2–3 weeks' pre-culture, which assures instant action when the experiment is set up.

The range of worker/larva ratio has been varied well beyond the limits known to occur in nature (BRIAN, 1950). Queen influence has been assessed in terms of survival to the pupal stage, progress towards pupation, the weight of pupae produced, and the caste of the pupae if female. All four are not usually manifest in the same instance.

VARIABLE WORKER/LARVA RATIOS

Larvae varied and workers constant

For this experiment *M. scabrinodis* was used. Two series, one with queens and one without, of 30 workers and 7, 9, 15, 22, 30, 45, or 60 larvae were cultured. The pupae yielded males, workers, and gynes (that is, queens in form but not in function), but as there were only 4 of the last they have been pooled with workers

TABLE 1—ANALYSIS OF THE PUPAE PRODUCED IN CULTURES WITH VARYING NUMBERS OF LARVAE AND THIRTY WORKERS EITHER IN THE PRESENCE OF A QUEEN (+) OR NOT (−): *M. scabrinodis*

No. of larvae	Queen status	Females			Males			Percentage of males
		No.	Mean weight (mg)	Difference	No.	Mean weight (mg)	Difference	
	+	6	2·55		1	5·90		
7	−	3	3·00	0·45	0	—		
	+	7	2·53		2	5·05		—10·2
9	−	7	2·84	0·31	0	—		
	+	10	2·59		1	5·30	−0·25	
15	−	11	3·67	1·08	1	4·00		
	+	10	2·27		2	4·20		
22	−	18	3·54	1·27	2	4·25		12·5
	+	27	2·94		2	5·35		
30	−	24	3·32	0·38	6	5·87		13·6
	+	21	2·68		5	4·90		
45	−	23	2·89	0·21	7	5·63	0·73	21·4
	+	35	2·52		12	4·50		
60	−	33	2·74	0·22	13	5·44	0·94	26·9
			Mean	0·56				

to give the column 'females' in Table 1. In this table the number of pupae produced and their average individual weights are given for males and females separately.

There is no doubt about a queen influence on females: the average weight of pupae was less in all cultures with queens than in the corresponding ones without (by analysis of variance, $P < 0{\cdot}01$). Thus, earlier findings are substantiated. The differences in average weight caused by queens vary considerably about a mean value of 0·56 mg per pupa but show no obvious simple regression relationship with the number of larvae. This indicates that the queen's influence cannot be diluted and a simple competitive relation with larvae is extremely unlikely.

Many fewer males were produced, but in four of the five pairs of cultures that can reasonably be compared they were smaller in the presence of a queen. For statistical analysis the five smallest groups were pooled giving mean pupal weights of 5·05 mg (with queens) and 5·30 mg (without queens) and these were then compared with the two largest groups (Table 1). Chance differences of this order were found to have a $P < 0{\cdot}001$. Thus the queen appears to act on male larvae as she does on female ones and we are entitled to draw the important inference that she influences the general physiology of growth and development in the larvae: she does not have a sex or caste specific action. An interesting further point that should be mentioned is that the difference due to the queen does regress on larval number (although this was not the case with females), suggesting at least that so far from diluting queen effect, a large larval population may enhance it.

Survival was similar with and without queens in this experiment. As would have been expected from earlier work, it was lower the more larvae there were (BRIAN, 1953). The proportion of males shows a steady rise from about 10 per cent to near 27 per cent, which may be taken to indicate, since larvae of both sexes were allocated to the cultures randomly, that male survival improves as competition with females intensifies. This result is difficult to relate to natural circumstances but it indicates none the less a possible experimental approach. (The regression is statistically significant, for it can be shown using χ^2 that the unordered proportions are heterogeneous and that the probability of an ordered sequence is only 1 in 60.)

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Workers varied and larvae constant

In the first of these experiments using *M. rubra*, two series of 10 larvae with either 5, 20, 35, 50, 65, 80, or 95 workers were set, one having two queens in each culture and the other no queens. After 24 days the brood stages were censussed and tabled (Table 2) and it was clear that there was again no difference in survival between the queen and no-queen treatments; the former had made substantially more progress. In those with queens nearly all the larvae had started to metamorphose but in those without, only a little over half had (this difference has $P < 0{\cdot}01$). Nevertheless, there is no sign of regression on worker number, perhaps because of the small number of larvae.

After 30 days when all brood change had ceased the experiment was concluded. Pupae were mainly worker and the few males that resulted have been pooled for calculation of average pupal weight per culture. This time the results have been graphed with regression lines drawn by eye (Fig. 1, the single and surely aberrant culture with 65 workers and no queen has been excluded).

It is clear that there is not a consistent queen effect this time and the results require further scrutiny. As would be expected from previous work (BRIAN, 1953) in the queen-less series the average weights increase with worker number up to a limit set by larval capacity, in this case about 3·8 mg with 35 workers. But in the series with queens they do not do so: there is no regression at all and all the weights lie within the small range $3{\cdot}4 \pm 0{\cdot}3$ mg. One may interpret the results by supposing that in some way (perhaps catalytically, perhaps by help with the work) the queen

is able to overcome a mild worker shortage but that this does not interfere with her size-reducing action.

TABLE 2—THE NUMBERS OF PUPAE PRODUCED FROM TEN LARVAE, AND THE NUMBERS OF RESIDUAL LARVAE AFTER A PERIOD OF CULTURE BY VARYING NUMBERS OF WORKERS EITHER WITH A QUEEN OR WITHOUT: *M. rubra*

Number of workers	Queen			No queen		
	Larvae	Pupae	Total	Larvae	Pupae	Total
5	1	9	10	6	3	9
20	0	10	10	4	6	10
35	0	7	7	4	2	6
50	2	7	9	1	8	9
65	1	8	9	2	8	10
80	1	9	10	4	6	10
95	0	9	9	3	5	8
Total	5	59	64	24	38	62

Before considering further the implications of this result, another similar experiment will be described. The same material was randomized and two new series set in which this time 20 larvae were cultured by either 5, 10, 15, 20, 25, 30, 40, or 60 workers; one series had two queens in each culture, the other none. The shift towards a lower worker/larva ratio in this series was aimed at a special

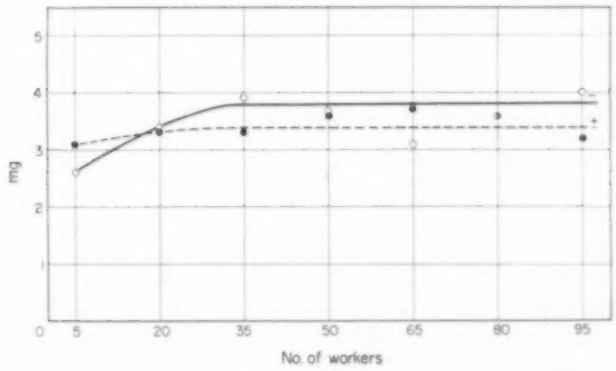

FIG. 1. The average weights of pupae derived from 10 larvae cultured by varying numbers of workers with or without queens; broken line, black circles, two queens; full line, white circles, no queen.

study of the regression zone. As before there was not a survival difference but nor was there a progress difference. Once again, the progeny were largely workers and their average weights per culture have been figured (Fig. 2) and regression curves drawn by eye (again one aberrant culture has been omitted).

The queen-less cultures regress better than last time and reach a maximum of 4·0 mg, only 0·2 mg greater. This time, however, the series with queens regresses at low worker numbers although the effect is much less than without

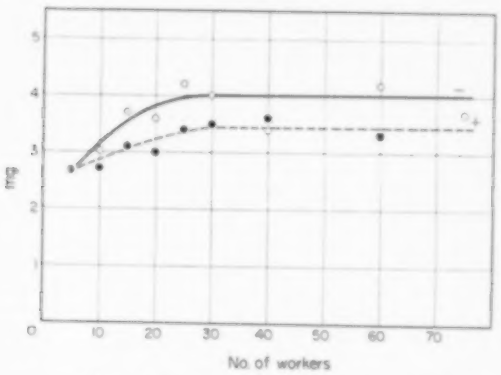

FIG. 2. The average weights of pupae derived from 20 larvae cultured by varying numbers of workers with or without queens; broken lines, black circles, two queens; full line, white circles, no queens.

queens; the value of the maximum is 3·45 mg, within 0·05 mg of last time. The magnitude of the queens' effect, 0·58 mg, is thus not much greater with 20 larvae than it was with 10 larvae, a noteworthy fact.

It seems justifiable to conclude from these experiments that the queen's reducing effect on adult or pupal size is small or non-existent in cultures in which the workers are unable to satisfy larval appetite (roughly ratios worker/larva less than 1). As workers become more numerous, the reducing effect increases but not indefinitely; a limit is reached when workers are super-abundant and larvae fully supplied with nutrients. Thus the queen reduces the ceiling not the threshold of size for pre-pupation.

An effect on survival

In all the above experiments no survival difference has been recorded. In concluding this section, therefore, it is proposed to give an example of this. Using *M. scabrinodis*, 6 workers and 5 larvae were given a queen in one set of 7 replicates and not in another. The experiment was repeated once. Using *M. rubra*, 6 workers and 6 larvae were given a queen in one set of 10 replicates and not in another set. The numbers of pupae resulting are given in Table 3. There is no doubt at all about the queen's effect on survival: these results have $P < 0{\cdot}001$. The fact that sometimes brood number is adjusted by mortality and sometimes not was noted in experiments on brood rearing by varying numbers of workers (BRIAN, 1953), and this appears to be a further case. Where adjustment of numbers cannot occur for some reason, perhaps because the larvae are well established, then the effect shows itself on size.

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TABLE 3—SURVIVAL TO THE PUPAL STAGE WITH AND WITHOUT QUEENS FOR TWO SPECIES OF *Myrmica*

	scabrinodis				*rubra*	
	Queen	No queen	Queen	No queen	Queen	No queen
Initial number of larvae	5	5	5	5	6	6
Final number of pupae	0	0	3	0	2	0
	5	0	3	3	6	3
	0	1	4	1	3	5
	3	0	0	0	0	0
	2	0	2	2	4	0
	3	0	0	0	4	0
	4	0			1	0
					3	3
					5	5
					3	2
Total	17	1	12	6	31	18
Replicates	7	7	6	6	10	10
Mean	2·4	0·1	2·0	1·0	3·1	1·8

DETAIL OF LARVAL GROWTH

Method

Single larvae have been cultured by workers with ample food with or without a queen and records have been made every other day of their weight, degree of maturity, and wing size. The larvae were in their final instar after hibernation and the workers were prepared for 2 weeks beforehand and used only once, whereas the queens were used several times (the reasons for this are explained in the last part of this paper). All cultures were kept at 23°C constant and 100 per cent r.h. Daily measurements were thought to be too disturbing and not necessary for the purpose in mind. The methods of measuring maturity and wing area have been described elsewhere (BRIAN, 1954). Briefly, maturity is based on a scale which depends on the fact that the brain moves slowly from the head into the thorax and that the leg buds then and only then begin to segment. For convenience the brain position is recorded in tenths of the total transit and the number of segments in the leg are recorded from 1 to 3 with half-way marks to indicate that the situation is transitional. After this the larva stops feeding, defaecates (and is then a pre-pupa), and moults into a pupa.

Altogether 128 larvae have been cultured. The data are difficult to summarize for females yield both gynes and workers, and the ontogenies of these are quite different. Within-caste grouping has therefore been unavoidable but it should be borne in mind that in the presence of queens, fewer gynes are produced. The production of males is of course free from this complication. The most useful comparisons are those of weight against time, weight against maturity, maturity against time, and wing against maturity. Others have been made but they are less

useful. Grouping for the first and third of these comparisons necessitates using a specific event as a fixed point and the most obvious one—the day on which culture starts—is not very useful since such larvae vary considerably in maturity. Better than this is the day when they stop feeding, but the best of all is a central time and the day on which the brain enters the thorax has accordingly been chosen and used throughout.

RESULTS

For this set of observations *M. rubra* was used. Each culture had 10 workers and 1 larva and there were three queens in each of one series and no queen in each of a control series. For a valid comparison of queen and no queen effects it is

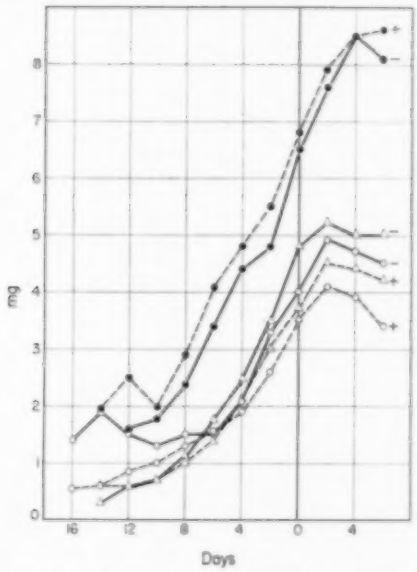

FIG. 3. The growth of single larvae with queens (+) or without (−); each curve an average of several cases. Time (abscissa) is in days from the stage of development when leg segmentation starts. ● Gynes; △ Males; ○ Workers.

necessary that the initial larval size frequencies of those reaching the pupal stage should be similar. This was not easily arranged for there was considerable mortality mainly of the smaller larvae in the queen-less cultures. The size frequencies of larvae are given in Table 4, both of those set to grow and of those that reached the pupal stage (and whose growth was accordingly recorded). For the reasons stated it is necessary to confine comparisons within each sex and caste.

(a) *Gynes*. From the graph of weight against time (Fig. 3) it may be noticed that to produce gynes in the presence of queens a little greater size is necessary at the start. The ultimate weights scarcely differ, however, and the general growth

Table 4—Initial size distributions of larvae in cultures with queens and cultures without; first all larvae and then those that survived to the pupal stage

Larval size (mg)	Queens		No queens	
	All	To pupae	All	To pupae
<0·6	36	27	53	21
0·6–1·0	5	4	3	2
1·1–1·5	3	2	2	2
1·6–2·0	7	6	7	5
2·1–2·5	7	6	5	4
Total	58	45	70	34

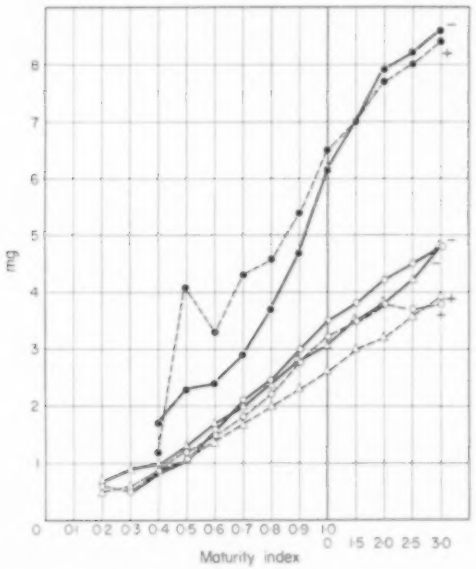

Fig. 4. The relation between the weight and the maturity of single larvae cultured with (+) or without (−) queens; each curve the average of several cases. ● Gynes; △ Males; ○ Workers.

rate is therefore less. The graph of weight against maturity (Fig. 4) shows that the larvae with queens get a good start but fail to maintain it. The maturity/time graph (Fig. 5) shows that rates of development are practically identical. The wing/maturity graph (Fig. 6) is interesting: the larvae with queens happen to begin with larger wings than the others but are overtaken at the start of the period of leg segmentation, so that, on the whole, the wings grow more slowly in the presence of queens.

(b) *Workers.* Larvae giving workers in the presence of queens start growing immediately instead of delaying but fail later to achieve as high a growth rate as those without queens. As a consequence they are smaller for most of the ontogeny and of course give smaller pupae. The rate of development is hardly different, rather slower if anything, in the period of leg segmentation. The wings are so small in this caste that comparison is meaningless.

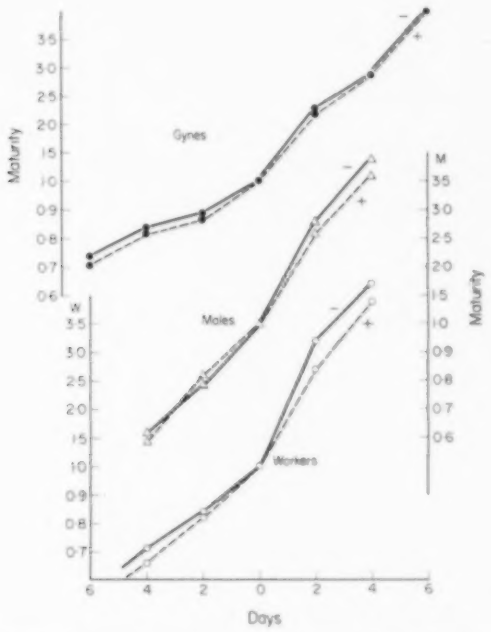

FIG. 5. The relation between maturity and time for single larvae cultured with (+) or without (−) queens; each curve the average of several cases; abscissa in days from start of leg segmentation. Each pair of curves has its own ordinate.

(c) *Males.* Male larvae in the presence of queens are at all times and stages of development smaller than in the absence of queens and their wings are smaller in the leg segmentation period. Their rate of development is not much different, slightly slower perhaps in the leg segmentation period.

One may reasonably conclude that in the presence of queens (1) there may be an earlier start to growth, but this advantage is soon lost, (2) growth of the body as a whole and of the wing is generally depressed in both sexes, (3) growth is depressed rather more in larger larvae, (4) rate of development is hardly affected, but if anything slightly depressed. Thus, once the initial conditions pass, the difference between larvae in cultures with queens and without, increases gradually. There is no sudden adjustment, no striking change of any kind, and the final difference in

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pupal weight is acquired gradually throughout ontogeny. This fits in well with the earlier results that indicated that the greater the possibilities for growth that the larva had (more workers each) the bigger the queen-effect. Finally, this result

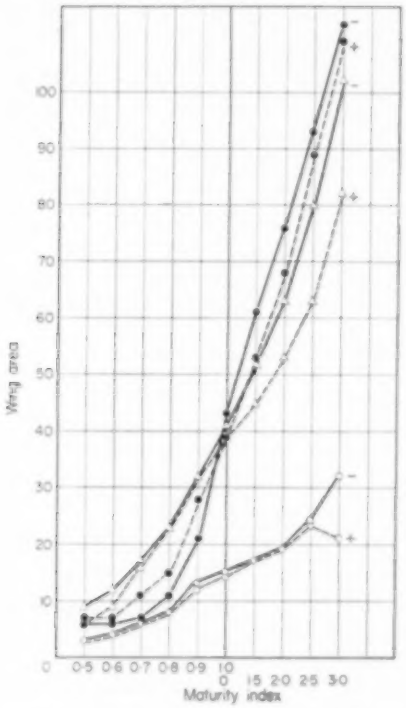

FIG. 6. Wing bud areas in relation to maturity in single larvae cultured with (+) or without (−) queens; each curve the average of several cases. Wing area is in arbitrary units of $6{\cdot}76 \times 10^{-4}$ mm^2. ● Gynes; △ Males; ○ Workers.

is achieved mainly by depressing growth rate, not by accelerating development rate. The earlier pupation in cultures with queens must result from the fact that larval growth is started earlier as a result of the queen's catalytic action.

QUEEN VARIABILITY

Suspicions that queens varied a good deal in their action were tested in the following way. Eight queens of *M. rubra* were given five successive trials, each time with freshly prepared workers. Control series were identical but for the absence of queens. From 1 to 6 larvae were reared to pupation in each trial, and the result assessed as numbers of gynes and workers rather than as the average weight. (There were no males this time.)

There is no doubt about a queen action in general (Table 5). Whereas in the presence of queens only 79 out of 140 females were gynes, in the control 130 out

TABLE 5—THE NUMBERS OF GYNE (G) AND WORKER (W) PUPAE PRODUCED IN FIVE SUCCESSIVE TRIALS BY EIGHT QUEENS EACH WITH TEN WORKERS, AND BY SIX GROUPS OF WORKERS WITHOUT QUEENS

Queen rank	Trials										Total	
	1		2		3		4		5			
	G	W	G	W	G	W	G	W	G	W	G	W
1	1	0	0	4	1	5	2	3	Dead		4	12
2	1	0	0	5	3	3	1	4	4	1	9	13
3	1	0	4	0	0	5	3	3	0	1	8	9
4	1	0	3	0	1	2	2	3	2	3	9	8
5	0	1	4	1	3	3	2	3	4	0	13	8
6	1	0	4	0	3	2	5	0	0	5	13	7
7	1	0	3	1	3	2	5	0	3	0	15	3
8	1	0	2	1	5	0	Dead		—	—	8	1
Total	7	1	20	12	19	22	20	16	13	10	79	61
Control (6 groups)	5	1	22	1	38	5	33	6	32	1	130	14

of 144 were (by χ^2, $P<0{\cdot}001$). There is also a suggestion that the power of the queen only develops after the first trial. The queens have been ranked according to their performance as gyne suppressors: the top one shows declining powers leading to death after four trials, the bottom one never shows any powers at all and died after three trials, whilst others are erratic.

TABLE 6—THE NUMBERS OF GYNE (G) AND WORKER (W) PUPAE PRODUCED BY A GROUP OF WORKERS FIRST WITH AN ACTIVE QUEEN THEN ALONE

Queen's rank	Trial number	Result			
		With queen		Alone	
		G	W	G	W
2	2	0	5	4	1
1	2	0	4	5	1
1	3	1	5	4	0
3	3	0	5	3	0
2	4	1	4	2	0
	Total	2	23	18	2

These observations were extended to give information about whether the workers that had been with an active queen carried her influence on to another trial although she was no longer with them. The top three queens were used and after a good performance the workers were taken and given larvae to culture. The results of five such tests are shown in Table 6, and it will be noticed that the trials are well distributed in time. There is no doubt that an active queen influence

is not carried with the workers, for they show a very much higher proportion of gynes in their yields alone than they do with the queens (by χ^2, $P < 0{\cdot}001$). Further, they give almost exactly the same ratio of gynes to workers 18/2 as do the control series over the whole experiment (18·6/2). Thus queen effect depends on queen-presence.

QUEEN NUMBER

In view of the above results it could be anticipated that several queens would be more likely to show a good effect than one alone, but this is not the same thing as saying that the queen effect is a function of their number. To test this last possiblity two experiments have been performed and will now be described.

TABLE 7—THE NUMBERS OF GYNE AND WORKER PUPAE PRODUCED FROM TWENTY LARVAE BY FORTY WORKERS OF *M. rubra* WITH EITHER NO, THREE, OR EIGHT QUEENS PRESENT; EACH TREATMENT DUPLICATED

Queen status	Replicate	Pupal caste		Average pupal weight (mg)
		Gyne	Worker	
0	1	19	0	6·97
	2	8	4	6·13
	Sum	27	4	6·55
3	1	4	16	4·58
	2	0	17	4·04
	Sum	4	33	4·31
8	1	7	10	4·97
	2	0	18	3·77
	Sum	7	28	4·37

First, groups of 20 workers and 10 larvae of *M. rubra* were cultured with either 0, 3, 6, or 12 queens (the first in triplicate) and the usual observations made. After 21 days all the larvae in cultures with queens had become pre-pupae or pupae, whereas in the three queenless cultures there were respectively 5, 3, and 5 larvae left, that is, only a little more than half had changed. This presents a strong contrast between cultures with queens and those without, but not between cultures with different numbers of queens. The average weights of pupae (which were all male and worker) were for the queenless cultures: 3·7 mg and for those with queens: 3·9 mg with 3, 3·6 mg with 6, and 3·3 mg with 12. These differences are not statistically significant, hence queen effect is only manifest here as a difference in rate of brood progress and it does not vary with the number of queens.

Secondly, groups of 40 workers and 20 larvae of *M. rubra* were formed with either 0, 3, or 8 queens. Two colonies were used to form duplicate series, and the experiment was left until all larvae had either died or pupated. The individuals were then weighed and grouped according to caste (all were female). Whereas a very high proportion of gynes to workers resulted in the absence of queens (Table 7), the reverse was the case when queens were present, that is, there is a striking queen effect; but there is not a significant difference (by χ^2) between the two sets of cultures with different numbers of queens. Again then, number does not increase effect. These results are expressed in the average weight data of the last column. In passing, it is interesting to notice that the replicates differed significantly (by χ^2, $P < 0{\cdot}001$), the first showing a greater tendency to give gynes.

These two experiments thus agree in indicating that queen effect is not augmented by an increase in queen number.

DISCUSSION

This work has shown that in the presence of a queen, the workers tend to wander away from the brood less and start feeding the larvae earlier and more regularly. Yet, as the larvae grow, they are more and more underfed and by the time they start to metamorphose they are definitely smaller than they could be. Often more survive when there is a queen so that in a sense the food is better distributed.

An interesting feature of the effect is that it requires super-abundant workers for its fullest expression. But the action is not a function of the worker number beyond this saturating stage and further workers are superfluous. Also, within fairly wide limits, there is no sign that with big larval populations the effect is less powerful and in fact there is evidence to the contrary. As might be expected, queens vary in their power, and more interesting, this appears to increase with culture; supposedly it is zero at the start of a season and increases throughout. However, multiplying the number of queens does not multiply their effect. Lastly, even if the workers carry the effect it does not persist with them for any length of time, and it seems that the queen's presence is essential.

One theory about the cause of the action which can be definitely ruled out is that it is due to competition between the queen and larvae for food; for if so, the more larvae there are, the less the effect would be and this is not so. Another theory that can be rejected is that the queen by improving survival increases larval competition and brings about the formation of more but smaller adults; but the effect on size is conspicuous even where there is no effect on survival.

Three interesting theories that cannot be ruled out are worth brief mention. First that the queen in some way causes the workers to neglect larvae more and more, the bigger they get. This would be an inversion of the state of affairs in queen-less groups where it has been shown that the larger larvae are favoured even to the complete exclusion of the small ones (BRIAN, 1957). It is often reported in the literature that when a colony becomes queen-less, there is an unusually high mortality, and effort is concentrated on a few larvae only (see BRIAN, 1958, for review). One way in which the queen might affect worker behaviour is by smearing

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the larger larvae with a mild repellent sufficient to reduce worker interest. We have noted that these larvae are prepared even to swallow air and that the workers only lick them cursorily and never seem to settle down to give them a long feed.

A second hypothesis is that the queen produces a growth-suppressive substance that is incorporated in the food. Since large larvae are normally given more food than small ones, even in proportion to their relative weight (Brian, 1957), the result would be a greater reduction in growth rate, the larger the larvae. This situation is rather interesting, for the queen first increases larval growth rate and then progressively impedes it, a control of negative feed-back type. An objection to this mechanism is that there does not appear to be a greater effect where larvae are few as might be expected, but this is not insuperable.

Finally, it is worth mentioning that the observed effects could arise entirely from increased worker activity. There is no doubt that they are activated as well as induced to concentrate on brood-rearing by the queen. This may be brought about through the queen's dominance, for she walks where she will and is always most sensitive to disturbance by vibration or light. If worker activity is increased, then larval stimulation by treading and licking will be increased and their respiration/assimilation quotient would be expected to rise. Under such circumstances it is also possible that the workers will collect more sugar and that the food regurgitated to larvae will have a low protein composition.

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EFFECT OF SUGARS ON FEEDING OF LARVAE OF THE SILKWORM, *BOMBYX MORI*

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Abstract—By applying an agar-diet containing starch, casein, leaf powder, cellulose powder, and sugar, the effect of various sugars and related compounds on the feeding of the silkworm was tested. Of more than twenty substances tested, including monosaccharides, disaccharides, trisaccharides, glycosides, and sugar alcohols, sucrose stimulated feeding most, followed by fructose and raffinose. Arabinose, rhamnose, galactose, maltose, melezitose, α-methylglucoside, and inositol were more or less stimulating. Glucose was a very weak stimulant for the silkworm. The effect of sucrose increased with increased concentration. The larva was further shown to be stimulated to feed by sucrose even when no leaf powder was added to the diet. However, the best feeding was obtained in the presence of both sucrose and leaf powder. Casein, at a high level, also stimulated feeding significantly, and the same was true for cellulose powder. Concerning the food specificity of the silkworm, the importance of the nutrients as feeding stimuli was emphasized. The feeding behaviour of the silkworm is briefly discussed.

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INTRODUCTION

THERE are many publications on rearing experiments of silkworm larvae, *Bombyx mori*, and on digestion and utilization of the components of mulberry leaves. The results obtained have contributed to improving practical techniques, such as rearing larvae or cultivating mulberry trees. However, the nutritional requirements of the silkworm can never be determined by such analyses. No attempt for devising a chemically-defined diet for this insect has hitherto been successful. YOSHIDA and his co-workers (unpublished data) were able to rear the larvae from hatching through the middle of the fifth instar, but never to maturity, on a diet containing over 50 per cent mulberry leaf powder, starch, sucrose, and soybean powder. It has been reported by OKABE (1952) that the silkworm larva does not feed at all on the diet prepared according to the formula described by BECK and STAUFFER (1950) for the European corn borer, *Pyrausta nubilalis*. Before formulating a suitable test diet, however, it seems to be necessary to establish the conditions required for feeding of the silkworm.

Attention has so far been paid to 'odd' chemical attractants in the study of host specificity of the silkworm, as previously done for other phytophagous insects. WATANABE (1958) demonstrated that two volatile compounds, α, β-hexenal and β, γ-hexenol, both of which were isolated from mulberry leaves, are the attractants for silkworm larvae.

Little is known on the mechanism of food selection of the silkworm. TORII and MORII (1948) found that the larva can perceive the odour of a mulberry leaf from a distance by means of the antennae, whereas the extirpation of maxillae results in feeding on some but not all of other leaves tested, which are not normally accepted. The failure of such ectomized larvae to eat certain plants led to the suggestion that there were other chemosensory organs besides the maxillae. This problem was re-investigated by ITO *et al.* (1959) with the result that there appeared to be organs of *taste* besides the maxillae concerned in continuous feeding. FRAENKEL (1959) has pointed out that the food specificity of the silkworm seems to be due to compounds recognized rather by taste than by smell. By feeding the silkworm on an agar-diet containing mulberry extracts, HAMAMURA (1959) has shown that there are two stimuli for biting and continuous swallowing, besides volatile compounds, in mulberry leaves; the former occurs in the methanol-soluble fraction of the leaves, the latter in the fraction insoluble in methanol and soluble in water. In view of these recent advances, the importance of taste is coming to be recognized in the food specificity of this insect, though it has not yet been fully demonstrated. Recently, several papers have been published emphasizing the significance of nutrients as feeding stimuli in phytophagous insects (BECK, 1956c; THORSTEINSON, 1958a, b).

Sugars have often been used for studies on the feeding and nutrition of insects. Sugar preferences in adult dipterous insects have been extensively investigated by many authors (VOGEL, 1931; VON FRISCH, 1934; HASLINGER, 1935; HASSETT *et al.*, 1950; WYKES, 1952; DETHIER and RHOADES, 1954; DETHIER *et al.*, 1956; GALUN and FRAENKEL, 1957; also see reviews by DETHIER, 1953; LIPKE and FRAENKEL, 1956), but scarcely in leaf-eating insects. The present study was undertaken to establish the responses to sugars and related substances in feeding behaviour of the silkworm. The results obtained indicated the importance of sugars as stimuli for feeding, as was previously recognized by THORSTEINSON (1958a).

MATERIALS AND METHODS

Newly moulted unfed fifth instar larvae of the silkworm were used in the present study. Four hybrids, crossed between stock lines, ((C110 × N112) × Daizô) × C115, N115 × C108, N112 × C110, and (N112 × C110) × Daizô, were chosen and will be referred to as hybrid A, B, C, and D, respectively. The larvae were starved for 1 day at 25°C prior to being placed on the diet, since a preliminary experiment had shown that they did not start feeding on the diet unless sufficiently starved.

The test methods used for feeding responses were principally based on the procedure described in the study on the chemotactic responses of the diamondback moth, *Plutella maculipennis*, by THORSTEINSON (1953), which were also recently applied in feeding tests of the silkworm (HAMAMURA, 1959). The agar-diet was prepared to meet possible nutritional requirements which have not yet been elucidated, and the composition of the test diet employed is shown in Table 1. In a preliminary experiment it was shown that the presence of either starch or

casein (HAMMARSTEN) did not reduce feeding. Agar and cellulose powder (General Biochemicals, Inc., Ohio) were considered at first to be inert carriers. Cellulose powder, however, was later shown to have a positive effect on feeding (Table 8). Approximately 20 mg of butyl-*p*-hydroxybenzoate was added to each Petri dish as a fungicide, without any noticeable effect on feeding. In some experiments the composition of the diet was partly changed, or one or two ingredients were omitted, as will be stated under 'Results and Discussion'.

TABLE 1—COMPOSITION OF THE DIET USED TO TEST THE EFFECT OF SUGARS ON THE FEEDING RESPONSES OF FIFTH INSTAR SILKWORM LARVAE

Substance	Amount used (per dish) (g)	Dry diet (%)
Agar	0·6	28·6
Starch	0·2	9·5
Sugar	0·4	19·0
Casein	0·2	9·5
Leaf powder	0·4	19·0
Cellulose powder	0·3	14·3
	2·1	99·9
Distilled water (ml)	20·0	

All the ingredients were mixed thoroughly by grinding in a mortar, and transferred into a Petri dish of 9 cm diameter with water. The mixture was heated to 95°C and then allowed to cool and gel. The gelled medium was cut into angular rods, which were put into the dish crosswise, since fifth instar larvae usually feed from the edges of leaves. Three larvae were placed on each diet and kept either at 25°C (spring and autumn) or at room temperature (mid-July).

Feeding responses were estimated by counting the number of faeces, as previously done by THORSTEINSON (1953). It is well known that there is a highly significant correlation between the amount of mulberry leaves ingested and the number of faeces produced. Since the number of faeces produced usually varied (Table 9), all the experiments were repeated from four to ten times. The experiments were carried out for 2 or, sometimes, 3 days and faeces counts were made per Petri dish, that is, per three larvae. The larvae were allowed to feed for 22 hr and, after 2 hr off the dish, transferred to a fresh diet. Counting was made on the combined faeces obtained during those 24 hr.

The mulberry leaves used were picked in the autumn, dried at room temperature, and kept in a desiccator. When necessary, dried leaves were pulverized. Leaf sap was prepared as follows: fresh leaves were homogenized in a Waring blendor with water (1 : 1, by wt.) and the homogenates strained through gauze layers. The solution obtained was heated to over 90°C for 5 min and, after cooling, centrifuged at 6000 rev/min for 15 min. The sap thus obtained was stored at −20°C until used.

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RESULTS AND DISCUSSION

Preference for sugars and related compounds

The effect of over twenty sugars and related compounds on feeding was tested, using two hybrids reared at different seasons. The results are summarized in Table 2. Faeces counts with hybrid A in summer were generally more than three

TABLE 2—EFFECT OF DIFFERENT SUGARS AND RELATED COMPOUNDS ON THE FEEDING OF FIFTH INSTAR SILKWORM LARVAE

Sugar or related compound	Mean faeces count per dish (Three larvae)			
	Expt. 1*		Expt. 2**	
	First 24 hr	Second 24 hr	First 24 hr	Second 24 hr
Pentoses				
L-Arabinose	3·0 ± 2·6†	9·3 ± 5·1	9·3 ± 5·8	20·8 ± 5·4
L-Rhamnose	5·3 ± 3·5	8·3 ± 4·1	6·3 ± 0·8	21·3 ± 3·4
D-Ribose	0	0·8 ± 0·4	2·8 ± 2·5	19·5 ± 7·0
D-Xylose	1·3 ± 0·8	1·5 ± 1·1	3·8 ± 1·5	15·8 ± 4·3
Hexoses				
D-Fructose	15·3 ± 8·2	17·8 ± 3·8	30·5 ± 7·6	47·8 ± 8·0
D-Galactose	0·3 ± 0·4	2·0 ± 0·7	6·8 ± 3·6	21·8 ± 9·0
L-Sorbose	0·8 ± 1·3	2·0 ± 0·7	6·3 ± 3·8	20·5 ± 9·7
D-Glucose	1·3 ± 1·1	3·8 ± 2·8	6·5 ± 4·4	14·8 ± 5·4
D-Mannose	2·0 ± 1·9	4·8 ± 2·9	1·5 ± 1·0	12·8 ± 5·1
Disaccharides				
Sucrose	17·8 ± 4·8	27·5 ± 7·0	40·0 ± 4·2	64·5 ± 8·1
Maltose	9·0 ± 4·2	11·3 ± 4·4	6·8 ± 3·8	20·5 ± 10·4
Lactose	1·5 ± 1·5	3·0 ± 2·9	3·0 ± 1·4	18·3 ± 6·5
Cellobiose	0·3 ± 0·4	1·3 ± 0·4	6·0 ± 5·8	15·8 ± 3·4
Trehalose	1·5 ± 1·1	3·8 ± 3·3	3·5 ± 2·7	12·5 ± 10·8
Melibiose	0·3 ± 0·4	3·0 ± 1·9	4·8 ± 2·1	11·5 ± 4·3
Trisaccharides				
Raffinose	10·0 ± 4·2	16·8 ± 4·6	9·8 ± 4·2	38·8 ± 8·3
Melezitose	2·0 ± 2·1	2·5 ± 1·1	4·8 ± 1·1	20·3 ± 1·5
Glycosides				
α-Methylglucoside	5·0 ± 1·6	5·5 ± 2.9	6·0 ± 3·3	23·0 ± 10·0
α-Methylmannoside	1·8 ± 1·9	1·8 ± 1·2	5·0 ± 5·1	15·8 ± 4·0
Sugar alcohols				
Inositol	—	—	13·8 ± 5·4	23·0 ± 6·4
Mannitol	—	—	5·3 ± 3·2	15·8 ± 3·7
Dulcitol	—	—	1·8 ± 0·8	14·8 ± 5·3
Sorbitol	1·0 ± 0·7	2·5 ± 2·3	2·8 ± 1·8	12·0 ± 5·6
Erythritol	—	—	1·8 ± 1·5	11·8 ± 3·3
Control–1§	2·0 ± 1·2	2·8 ± 1·9	3·8 ± 3·1	12·3 ± 5·5
Control–2	—	—	0·3 ± 0·4	1·8 ± 1·1
Agar alone	1·3 ± 1·3	1·0 ± 0·7	0·5 ± 0·5	2·8 ± 0·8

Number of replicates is four.

* Hybrid B (spring rearing). ** Hybrid A (summer rearing).

† Standard error.

§ Control–1 diet contained all the ingredients but sugar, control–2 diet contained all but sugar and leaf powder.

times (ranging from two to ten times with different sugars) as much as those with hybrid B in spring. This is partly due to the fact that more food was ingested at the higher temperature. However, faeces counts never doubled even when the temperature was increased from 20 to 30°C (ITO, unpublished data). Furthermore, the difference in room temperature between spring (25°C) and mid-July was approximately 5°C. Consequently, the differences in faeces counts between two hybrids may be attributed to 'racial difference' in feeding responses.

Before moulting (the fourth moulting period lasts approximately 24 hr at 25°C), the larva empties nearly all foodstuffs from the gut lumen. The little retained material is usually evacuated as one or two faeces during starvation after the moult. Faeces obtained on agar-gel alone were of this nature, since this gel was consistently unacceptable for silkworm larvae. This was also so on a diet devoid of leaf powder and sugar (control–2). Feeding was induced in the presence of leaf powder, even without sugar (control–1), and the faeces count thus obtained was taken as the control for evaluating feeding responses for sugars.

At a sugar level of 19·0 per cent per dry diet the feeding responses differed greatly for different sugars (Table 2). The most stimulating effect was obtained with sucrose, followed by fructose and raffinose in this order of effectiveness, with consistently high significance. The responses for arabinose, rhamnose, maltose, α-methylglucoside, and inositol were positive, but not always significantly so. Galactose and melezitose seemed sometimes to improve feeding. Other sugars used, including glucose, had a weak effect or were inert. None of the sugars seemed to have a negative effect. The studies on sugar preferences in dipterous insects (see the literature cited above) have shown that the most stimulating sugars are fructose, glucose, and fucose of the monosaccharides, and sucrose and maltose of the disaccharides. Silkworm larvae reacted similarly, except for glucose; fucose was not available in the present study. As for raffinose, dipterous insects have been reported to react differently according to species. Xylose, mannose, and melibiose were preferred neither by the silkworm nor by dipterous insects. Although there are some differences in the responses for sugars between the silkworm and dipterous insects, there exist also many similarities.

From the results in Table 2, there seems to be no suggestion in the silkworm that the first step in the stimulative action of the disaccharides involves their hydrolysis, since stimulation by glucose alone was very weak, whereas maltose ($\frac{1}{2}$ glucose + $\frac{1}{2}$ glucose) showed a positive response, and fructose was less stimulatory than sucrose ($\frac{1}{2}$ glucose + $\frac{1}{2}$ fructose) at the same concentration.

The mean faeces count produced during the first 24 hr was always less than during the following 24 hr. This includes at least two possibilities: (1) the larvae ingested more food during the second 24 hr than during the first 24 hr, as when reared on leaves (for instance, ITO and TANAKA, 1959b; cf. Table 9); (2) the larvae became adapted to the diet on the second day. This phenomenon is not obvious with the more stimulating sugars present, but is evident with the less stimulating ones, as judged from a marked increase in faeces counts on the second day. The larva of hybrid A is more readily adaptable than that of hybrid B.

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Only a few papers stress the importance of sugars in the feeding behaviour of phytophagous insects. BECK (1956b) found dietary sugars (glucose, fructose, and sucrose) to have a marked influence on larval feeding behaviour of the European corn borer. An inducing effect of sucrose on feeding was also reported for the grasshopper, *Chorthippus linbicornis*, by THORSTEINSON (1958a), who further emphasized the fact that the insects not only accept sucrose but are also induced to feed by it (1958b). The results presented here support those previous observations, though some differences exist as to the kinds of sugars. The most outstanding difference was the response to glucose, which was preferred by dipterous insects, and has also been used as one of the ingredients in the diets for several plant-feeding insects (BECK and STAUFFER, 1950; BECK, 1956b; HIRANO and ISHII, 1957; SHEEL *et al.*, 1958).

The fact that glucose is rather non-stimulative for feeding of the silkworm is also evident from the tests on the effect of sugars at varying concentrations. The results of this experiment are shown in Tables 3 and 4 for sucrose and glucose,

TABLE 3—EFFECT OF SUCROSE CONCENTRATION ON THE FEEDING OF FIFTH INSTAR SILKWORM LARVAE (SUMMER REARING)

Amount of sucrose per dish (g)	Amount of sucrose per dry diet (%)	Faeces count per dish (Three larvae)			
		Expt. 1*		Expt. 2**	
		First 24 hr	Second 24 hr	First 24 hr	Second 24 hr
0	0	1·0 ± 0·0†	9·3 ± 4·3	5·0 ± 0·5	7·3 ± 3·8
0·04	2·4	2·5 ± 0·9	11·5 ± 6·3	4·5 ± 1·1	7·0 ± 4·1
0·1	5·9	9·8 ± 4·0	28·8 ± 5·2	5·0 ± 2·9	17·3 ± 5·9
0·2	11·1	16·0 ± 6·6	42·8 ± 8·2	4·3 ± 2·4	24·0 ± 7·7
0·4	20·0	19·0 ± 5·2	40·8 ± 4·0	10·0 ± 5·2	27·0 ± 5·4
0·6	27·3	25·0 ± 6·9	42·8 ± 7·8	7·8 ± 3·8	24·5 ± 6·6
0·8	33·3	28·3 ± 10·2	44·3 ± 7·3	10·0 ± 6·5	28·0 ± 7·4

Number of replicates is four.
* Hybrid A.
** Hybrid C.
† Standard error.

respectively. Here the amount of leaf powder was reduced from 0·4 to 0·2 g per dish and that of cellulose powder increased from 0·3 to 0·4 g. The feeding was increased with increasing sucrose content of the diets. However, a plateau was reached at 11·1 per cent sucrose per dry diet, as far as the feeding during the second 24 hr was concerned (Table 3). No negative effect on feeding at the highest concentration tested was observed. Contrary to the result on sucrose, stimulation by glucose was very low even at extremely high concentrations, and no significantly positive responses were consistently observed with increasing levels of glucose (Table 4). Insects have been reported usually to prefer the higher concentration of sugars including glucose rather than the lower one (WYKES, 1952; DETHIER and RHOADES, 1954; DETHIER *et al.*, 1956; BECK, 1956a, c; GALUN and FRAENKEL, 1957).

From Table 3 it is obvious that the stimulating effect of sucrose is more evident in hybrid A than in hybrid C. Hybrid A originated by successive crossing between silkworm lines, one of which is a multi-voltine race, Daizô. Though no data are available on feeding behaviour in relation to voltinism, it is evident that racial differences in feeding behaviour must not be overlooked.

TABLE 4—EFFECT OF GLUCOSE CONCENTRATION ON THE FEEDING OF FIFTH INSTAR SILKWORM LARVAE

Amount of glucose per dish (g)	Amount of glucose per dry diet (%)	Faeces count per dish (Three larvae)		
		Expt. 1*		Expt. 2**
		First 24 hr	Second 24 hr	Second 24 hr
0	0	2·0 ± 1·2†	7·0 ± 5·2	2·8 ± 0·4
0·1	5·9	2·5 ± 1·7	5·5 ± 4·4	3·0 ± 0·7
0·4	20·0	2·0 ± 1·2	2·3 ± 0·7	4·8 ± 1·7§
0·8	33·3	4·3 ± 1·3	13·0 ± 2·7	8·8 ± 2·4
1·4	46·7	2·3 ± 0·9	9·0 ± 6·4	10·5 ± 3·5
2·0	55·6	3·0 ± 0·7	7·0 ± 6·0	9·5 ± 2·7
4·0	71·4	6·0 ± 3·1	20·5 ± 5·4	—

Number of replicates is four.
* Hybrid A (summer rearing).
** Hybrid D (late autumn rearing).
† Standard error.
§ Mean faeces count, 29·3 ± 10·6, was obtained on sucrose diet (0·4 g per dish), performed at the same time with the same hybrid.

TABLE 5—EFFECT OF VARYING CONCENTRATIONS OF BOTH SUCROSE AND GLUCOSE ON THE FEEDING OF FIFTH INSTAR SILKWORM LARVAE (SUMMER REARING)

Amount of sucrose per dish (g)	Amount of glucose per dish (g)	Amount of sucrose per dry diet (%)	Mean faeces count per dish (Three larvae*)	
			First 24 hr	Second 24 hr
0·4	0	19·0	40·9 ± 10·2†	58·9 ± 9·6
0·3	0·1	14·3	40·0 ± 9·5	47·4 ± 9·1
0·2	0·2	9·5	23·0 ± 8·1	36·4 ± 9·3
0·1	0·3	4·8	24·4 ± 12·1	27·4 ± 10·4
0	0·4	0	2.8 ± 1·9	9·2 ± 3·7

Number of replicates is eight.
* Hybrid A.
† Standard error.

When sucrose and glucose were present in mixtures of varying concentrations, the response was rather similar to those when sucrose alone was present (Table 5), though there seemed to be a slight additive effect. This indicates again that glucose is rather inert for feeding stimulation.

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Since sucrose was shown to be a conspicuous stimulus for feeding of the silkworm, the question arose as to whether sucrose may replace leaf powder as a feeding stimulus. This problem was subsequently investigated by varying the amounts of either leaf powder in the presence or absence of sucrose, or of sucrose in the absence of leaf powder. The amounts of leaf powder, sucrose, and cellulose powder varied as shown in Table 6; other ingredients were added as shown in

TABLE 6—EFFECT OF SUCROSE AND LEAF POWDER ON THE FEEDING OF FIFTH INSTAR SILKWORM LARVAE (SUMMER REARING)

Amount of leaf powder per dish (g)	Amount of sucrose per dish (g)	Amount of cellulose powder per dish (g)	Faeces count per dish (Three larvae, hybrid A)		
			Expt. 1*	Expt. 2**	
			First 24 hr	First 24 hr	Second 24 hr
0·6	0	0·2	19·8 ± 4·5†	10·0 ± 4·2	16·7 ± 4·1
0·4	0	0·4	17·8 ± 2·9	4·0 ± 1·6	13·5 ± 3·9
0·2	0	0·6	9·3 ± 4·8	3·3 ± 1·2	11·5 ± 5·0
0	0	0·8	4·0 ± 2·6	2·0 ± 0·0	3·3 ± 0·4
0·6	0·4	0·2	41·5 ± 5·9	65·9 ± 5·3	77·5 ± 7·3
0·4	0·4	0·4	36·5 ± 6·0	55·2 ± 9·9	70·3 ± 17·3
0·2	0·4	0·6	29·5 ± 2·2	18·5 ± 4·7	38·8 ± 10·4
0	0·8	0·2	17·3 ± 1·7	18·0 ± 5·3	32·5 ± 6·6
0	0·6	0·4	17·8 ± 7·3	13·0 ± 7·4	27·8 ± 8·7
0	0·4	0·6	17·8 ± 5·5	23·3 ± 3·6	21·0 ± 6·6

Number of replicates is four.
* Starved for 2 days prior to the experiment.
** Starved for 1 day as usual.
† Standard error.

Table 1. A clear-cut correlation between feeding response and concentrations of leaf powder was obtained in the absence of sucrose (Table 6). THORSTEINSON (1953) has reported a similar effect in regard to leaf powder concentration in the diamondback moth. Table 6 also shows that the response is markedly increased when sucrose is added to leaf powder. It is of interest that the feeding response was highly positive in the presence of sucrose alone, without leaf powder. Faeces counts in Table 6 indicate that sucrose alone is more stimulative than leaf powder alone. The same pattern in regard to sucrose has been recognized in the Colorado potato beetle, *Leptinotarsa decemlineata* (THORSTEINSON, 1958a). However, it is impossible to conclude that mulberry leaf powder can be entirely replaced by sucrose, since an increased effect was induced by the combination of those two. Such an effect of the combination is rather similar to a finding by HAMAMURA (1959), according to which a full feeding response is obtained by adding a methanol-soluble fraction of mulberry leaves to a water-extract of the residue, though each fraction alone stimulates feeding only slightly.

Other factors influencing feeding

Since the diets used in the present study contain casein, starch, and cellulose powder, tests were carried out to determine the effects of these three substances. Those are shown in Tables 7 and 8. The composition of the diets used for those tests was essentially the same as the basic test diet, except for the compounds listed in Tables 7 or 8. Both casein and starch were not consistently stimulative for

TABLE 7—EFFECT OF CASEIN AND STARCH ON THE FEEDING OF FIFTH INSTAR SILKWORM LARVAE (AUTUMN REARING)

Amount of sucrose per dish (g)	Amount of casein per dish (g)	Amount of starch per dish (g)	Mean faeces count per dish (Three larvae*)	
			First 24 hr	Second 24 hr
0	0	0	1·1 ± 0·7†	4·2 ± 3·2
0	0·2	0	2·3 ± 2·2	9·3 ± 6·5§
0	0	0·2	2·3 ± 2·2	8·6 ± 4·6§
0	0·2	0·2	1·7 ± 1·9	8·8 ± 3·3‖
0·2	0	0	19·9 ± 5·0	42·7 ± 10·3
0·2	0·2	0	22·4 ± 4·4	48·9 ± 5·1
0·2	0	0·2	17·6 ± 4·5	39·8 ± 9·7
0·2	0·2	0·2	27·2 ± 5·4‖	48·1 ± 6·5
Number of replicates is ten.				
0·2	0	0·2	8·9 ± 4·4	25·3 ± 9·6
0·2	0·2	0·2	13·6 ± 5·5	26·6 ± 8·7
0·2	0·5	0·2	26·9 ± 7·4‖	39·8 ± 10·6§

Number of replicates is eight.
* Hybrid A.
† Standard error.
§ Significant at 5 per cent.
‖ Significant at 1 per cent.

feeding, in the presence of sucrose and leaf powder. However, a highly positive response for feeding was obtained with increasing concentrations of casein. Stimulating effectiveness by casein on a weight basis was far less than that by sucrose. The stimulation to feeding by protein (albumin) has been referred to by THORSTEINSON (1958a).

For the test on the role of cellulose powder for feeding, two series of diets were prepared, one containing leaf sap, the other leaf powder, the other ingredients being agar and sucrose (0·4 g per dish). As shown in Table 8, a positive effect was induced by cellulose powder only in the presence of leaf sap, but not in the presence of leaf powder, which contains much cellulose. It was reported that cellulose powder is necessary for feeding of the European corn borer (BECK and HANEC, 1958).

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Table 9 shows the individual variation in faeces counts produced on the complete basic test-diet (Table 1) and on fresh mulberry leaves. Faeces counts varied even on leaves, but more greatly on agar-diet.

TABLE 8—EFFECT OF CELLULOSE POWDER ON THE FEEDING OF FIFTH INSTAR SILKWORM LARVAE (AUTUMN REARING)

Amount of cellulose powder per dish (g)	Mean faeces count per dish (Three larvae*)		Remarks
	First 24 hr	Next 19 hr	
0	20·8 ± 5·1†	15·9 ± 4·3	41·4 mg leaf sap concentrate (dry wt.) added instead of leaf powder
0·6	27·6 ± 7·6§	22·9 ± 5·9‖	

	First 24 hr	Second 24 hr	
0	7·2 ± 3·5	13·6 ± 4·2	0·2 g leaf powder added
0·4	8·6 ± 3·3	15·0 ± 4·1	

Number of replicates is ten.
* Hybrid A.
† Standard error.
§ Significant at 5 per cent.
‖ Significant at 1 per cent.

TABLE 9—INDIVIDUAL VARIATION OF FAECES COUNTS ON AGAR-DIET OR MULBERRY LEAF (SUMMER REARING)

Food	Mean faeces count per larva*		Number tested
	First 24 hr	Second 24 hr	
Agar-diet	18·6 ± 7·8†	—	30
	14·3 ± 9·8	30·5 ± 8·5	25
Mulberry leaf	36·2 ± 4·1	50·7 ± 5·0	20

* Hybrid A.
† Standard error.

Food specificity of silkworm larva

Mulberry leaves are generally considered to be the sole food for silkworm larvae, not only for best larval growth and viability, but also the best cocoon production. Most writers assume unique relation between the silkworm and mulberry leaves. The data so far obtained have shown that a number of leaves other than mulberry, most of them Moraceae, can substitute for mulberry for longer or shorter periods (summarized in the books by ITAYA, 1936; TANAKA, 1943;

reviewed by FRAENKEL, 1959; ITO, 1959), and it has long been believed that the odour of mulberry leaves plays the most important role in food selection.

Odour is, as a matter of fact, the important initial stimulus, as demonstrated by TORII and MORII (1948), showing that the antennae participate in distinguishing the smell. Of a number of compounds either extracted from mulberry leaves or other possible attractants, α, β-hexenal and β, γ-hexenol, steam distillates of mulberrry leaves, were shown to attract the silkworm (WATANABE, 1958). Those compounds are, however, widely distributed in leaves from various sources (CURTIUS and FRANZEN, 1912; TAKEI *et al.*, 1934, 1938). The discrepancy between the monophagous (or oligophagous ?) habit of the silkworm and the wide distribution of such volatile material has been pointed out also by FRAENKEL (1959). Maxillectomized larvae fed on some, but not all, of the wrong plants for a short period (TORII and MORII, 1948), but no continuous feeding was observed (ITO *et al.*, 1959). The opinion that taste is important for feeding is also supported by the study of the feeding behaviour of mutants showing morphological anomalies of the maxillae (TAZIMA, personal communication). Feeding on wrong plants after such extirpations is explained by the abolition of reception of repellent substances (FRAENKEL, 1959). In this connexion, a report by HAMAMURA (1959) indicating the presence of separate biting and swallowing stimuli is interesting.

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As referred to in an earlier part of this paper, the importance of token stimuli has hitherto been emphasized in the food selection of this insect. Those substances may attract larvae as olfactory stimuli that induce the insect to approach the leaves and may induce biting, but not feeding. The experiments on the effect of extirpation of the maxillae indicate that a gustatory mechanism is also involved in the feeding behaviour of the silkworm. From the present results, it is evident that feeding is stimulated by the nutrients in the food, which are odourless. The fact that a number of sugars stimulate feeding, and that the larva feeds on the diet containing sucrose alone better than that containing leaf powder alone, suggests that those nutrients are important in feeding behaviour of the silkworm, sometimes rather more important than token stimuli. This does not mean the less emphasis of token stimuli, since the combination of leaf powder and sucrose enhanced stimulation, and since leaf powder has not so far been replaced by sucrose in such an enhancement.

Finally, the relation between feeding and nutritive value of sugars in the silkworm will be mentioned briefly. Much work on insects has failed to establish a correlation between them. In the silkworm nothing has been known of such a relationship. Recently, however, a technique employing oral administration of the nutrients has been developed with considerable success (ITO and TANAKA, 1959a). The same technique has been applied for a variety of sugars, and it was recognized that there is no relation between sugar preference and nutritive value (ITO and TANAKA, 1960).

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ADDENDUM

Rearing of silkworm larvae entirely on an artificial diet has recently been achieved (FUKUDA *et al.*, 1960; ITO and TANAKA, 1960; YOSHIDA *et al.*, 1960). The composition of the diet used by FUKUDA *et al.* is entirely the same as that devised by YOSHIDA *et al.*, but that employed by ITO and TANAKA is slightly modified. Main constituents of those diets are starch, sucrose, soybean powder, and mulberry leaf powder. Growth was suboptimal on these diets.

ITO (1960) subsequently improved the diet by replacing crude products with possibly pure compounds and demonstrated that dietary soybean oil is required for moulting and growth in this insect.

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A COMPARATIVE STUDY OF THE EFFECTS OF DENERVATION ON THE ABDOMINAL MUSCLES OF SATURNIID MOTHS DURING PUPATION

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Abstract—In *Antheraea pernyi*, *Actias selene*, and *Samia cynthia* the pattern of persisting longitudinal muscles in the pupal abdomen is constant. The dorsal, lateral, and ventral bands are complete in the fourth, fifth, and sixth segments. In the third segment the ventral band is missing, the lateral is present, and the dorsal lacks the most ventral of its three sub-divisions. In all three species denervation of the muscles of the third segment by removal of the second or third abdominal ganglion from the spinning larva causes them to degenerate in the pupa. In *A. pernyi* the ventral third of the dorsal band of the fourth segment also degenerates. The result is the same whether the pupa enters diapause or continues to develop. Only in *A. selene* can all the muscles of the fourth and fifth segments be induced to degenerate by denervation, but even in this species degeneration is not complete. The muscles of the sixth segment are more sensitive to denervation. These results are additional proof of the existence of two gradients, an anterior and a posterior, that influence the degeneration of the abdominal muscles, so that muscles of the third and sixth segments are more readily induced to degenerate by denervation than those of the fourth and fifth segments. Experiments in which various nerves, including the median, were cut showed that no nerve was exclusively responsible for this trophic influence.

INTRODUCTION

THE pupa of a moth retains some of the larval longitudinal muscles of the abdomen. In a previous paper (FINLAYSON, 1956) are described the effects of denervation during the late larval or prepupal stage on the persisting muscles of the fourth, fifth, and sixth abdominal segments. It was found that denervation of these muscles in the waxmoth *Galleria mellonella* caused them to degenerate during the larval–pupal transformation but that in the saturniids *Antheraea pernyi* and *Hyalophora* (=*Platysamia*) *cecropia* the muscle pattern of the pupa was unaffected. The denervated muscles of the saturniids atrophied after a long period, but this atrophy was obviously brought about in a different way from the rapid degeneration of the muscles of *Galleria* that took place during the period of histolysis at metamorphosis. It resembles the atrophy of denervated muscles in the cockroach (ROEDER and WEIANT, 1949; NOVOTNÝ, 1958). It was suggested that the onset of diapause in the saturniids in some way halted the degeneration of the denervated muscles before the two waves of histolytic influence had reached them. These waves pass from anterior to posterior and vice versa. In the case of the longitudinal muscle bands they normally stop in the second and the seventh

abdominal segments, leaving intact longitudinal muscle bands in the third, fourth, fifth, and sixth abdominal segments.

The experiments described in the present paper were undertaken in an attempt to resolve or explain the differences between the responses of the waxmoth and the saturniids to muscle denervation.

MATERIAL AND METHODS

The species of Saturniidae used in this study were *Antheraea pernyi*, *Antheraea polyphemus*, *Antheraea yamamai*, *Actias selene*, and *Samia cynthia*. The strain of *S. cynthia* is a non-diapausing one, obtained by Mr. L. HUGH NEWMAN from Malta. It has many of the characteristics of *Samia cynthia ricini*. Even when it is reared in a short-day photoperiod (8 hr) it does not diapause. All species of the moths were reared in the laboratory at 25°C.

Surgical operations were carried out on spinning larvae anaesthetized with carbon dioxide. The wound was sealed as well as possible with molten paraffin wax. The use of phenylthiourea (FINLAYSON, 1956) was discontinued because it was found that the larvae survived as well without it. There is a very low mortality among larvae from healthy cultures after operations of the type performed in this investigation. After surgery each larva was placed in a small plastic box and kept at 25°C. The larval–pupal moult usually took place in less than a week and they were allowed to develop for about a week before they were dissected. The experiments carried out with *S. cynthia* are typical. The average period from operation to pupation was 5 days (range 3–6) and from pupation to dissection 6·8 days (range 5–12). The abdominal muscles of one side were inspected in the living animal and then after fixation in Bouin's fluid. The amount of muscle present was scored on the scale used previously (FINLAYSON, 1956): a score of 4 means normal full-sized muscle fibres, a score of 0 means that the muscles have completely degenerated leaving at most only flimsy vestiges. This scale is by no means precise but it is sufficiently accurate for assessing the results of the experiments described in the following sections.

NORMAL PATTERNS OF LONGITUDINAL MUSCLES OF PUPAL ABDOMEN

In my previous paper (FINLAYSON, 1956) I described the normal variation in the pattern of the persisting muscles of the third abdominal segment of *Antheraea* (=*Telea*) *polyphemus*. Because the musculature in this segment is variable, it was purposely disregarded in subsequent work on the effects of denervation. Dissections of 20 normal pupae of *A. pernyi*, 32 *A. selene*, and 23 *S. cynthia* have shown, however, that the muscle pattern of the third abdominal segment of these species does not vary (Fig. 1). The muscle fibres of the longitudinal bands in the third abdominal segment of the pupa of *S. cynthia* are thinner than those in the fourth, fifth, and sixth segments (scored as stage 3 of the scale). The ventral band is never present in the third segment and is omitted from consideration in the

experiments involving the fourth, fifth, and sixth segments because it may be damaged during surgical operations.

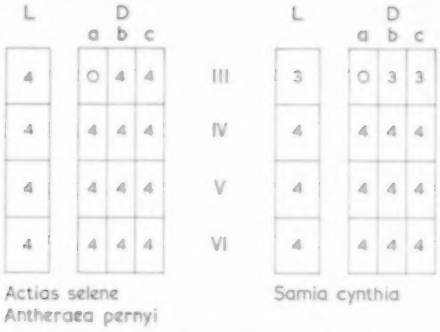

FIG. 1. Diagrams to show the pattern of the lateral and dorsal longitudinal muscles in the normal pupa of *Actias selene*, *Antheraea pernyi*, and *Samia cynthia*.

III–VI, abdominal segments; L, lateral muscle band; D, dorsal muscle band; a, b, and c, sub-divisions of dorsal band; 4, normal muscle; 0, completely degenerated muscle.

COMPARISON OF DIAPAUSING AND NON-DIAPAUSING ANIMALS

In order to test the hypothesis (FINLAYSON, 1956) that the onset of diapause influences the response of muscles to denervation, experiments were carried out on diapausing and non-diapausing saturniids. The species used were *Actias selene*, which has no diapause, *Antheraea yamamai*, which has an egg diapause, *Antheraea pernyi*, reared in a long photoperiod or a short photoperiod to produce non-diapausing and diapausing pupae respectively, and the non-diapausing strain of *Samia cynthia*.

In all these species and in both stocks of *A. pernyi* removal of the appropriate ganglia from the last instar larva led to the degeneration of muscles that normally persist in the pupa. Details of the operations and results will be given in later sections of this paper. At this stage I wish merely to eliminate diapause as a possible factor. The variation between species that was discovered in previous studies (FINLAYSON, 1956) cannot be correlated with the incidence of diapause. For example, the obligatory non-diapausing strain of *S. cynthia* is no more affected by denervation than *A. pernyi*, which responds in precisely the same way whether it is entering diapause or continuing to develop into the adult without interruption.

DENERVATION OF THE MUSCLES OF THE THIRD ABDOMINAL SEGMENT

After it was discovered that the pattern of the muscles in the third abdominal segment of the pupa of three species was constant, experiments were carried out to determine the effect of denervating these muscles. The third abdominal ganglion was removed from larvae of *A. pernyi* (19 survivors), *S. cynthia* (10 survivors), and *A. selene* (13 survivors). In *A. selene* all the nerves from the third ganglion (including the branches of the median of the second ganglion) were severed in another experiment (14 survivors).

In *A. pernyi* the pupae produced from the operated larvae invariably showed a reduction or complete degeneration of some muscles. There was considerable variation in the degree of degeneration. The animal that was least affected showed no abnormal degeneration in the third segment but in the fourth segment subdivision (a) of the dorsal band had completely degenerated. In all the others the

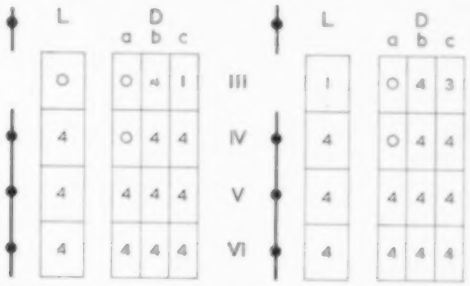

FIG. 2. Diagrams to show the effect of removing the third abdominal ganglion of the spinning larva on the lateral and dorsal muscle bands of the pupa of *Antheraea pernyi*.
III–VI, abdominal segments; L, lateral muscle band; D, dorsal muscle band; a, b, and c, sub-divisions of the dorsal band; 4, normal muscle; 0, completely degenerated muscle; other numbers represent degrees of degeneration.

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lateral band in the third segment had at least partially degenerated and subdivision (a) of the dorsal band of the fourth segment had completely degenerated (Fig. 2). In no animal had the longitudinal muscles of the third segment completely degenerated.

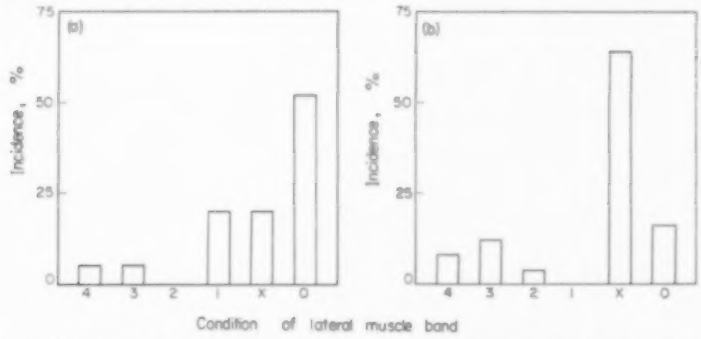

FIG. 3. Histograms showing the effect in (a) 19 *Antheraea pernyi* and (b) 26 *Actias selene* of removing the third abdominal ganglion of the spinning larva on the lateral muscles of the third abdominal segment of the pupa.
4, normal muscle; 0, completely degenerated muscle; other numbers represent degrees of degeneration; X, special category in which a number of fibres of normal or near-normal size have persisted in an otherwise completely degenerated band.

In *A. selene* removing the third ganglion or severing the nerves in the vicinity of the third ganglion also led to the degeneration of some of the longitudinal muscles of the third segment in all except 2 of the 26 animals. In the majority the lateral band had degenerated except for a few normal fibres (Fig. 3, X). About

half the animals had normal dorsal muscles and the other half showed incomplete degeneration (Fig. 6). The middle sub-division (b) of the dorsal band of those animals showing degeneration was less reduced than the other two sub-divisions in all except one animal in which the entire dorsal band had gone. In *A. selene* the lateral band suffered greater reduction than the dorsal band following the loss of innervation from the third ganglion. The dorsal band of the fourth segment was normal in every animal but in 3 animals there was some degeneration in the lateral band. In 2 of these animals there was only a thinning of the fibres of the lateral band to stage 3, but in one of them some fibres had degenerated completely and the rest were of normal thickness.

In an experiment with *A. selene* the third and fourth abdominal ganglia were removed. In the 13 pupae obtained there was total degeneration of the longitudinal muscles of the third segment in 11 animals and greatly reduced muscles in 2 animals. This result is the only one that indicates a reinforcement of a ganglion by its more posterior neighbour in the nerve cord.

In *S. cynthia* removal of the third abdominal ganglion always leads to extreme degeneration of the longitudinal muscles of the third abdominal segment. In all 10 animals the lateral band had degenerated and in the majority sub-division (c) of the dorsal band had completely degenerated. In 5 animals all the longitudinal muscles had completely degenerated. In no animal was the fourth segment affected.

A. pernyi is therefore the only species of saturniid among those studied in which removal of the third ganglion regularly results in degeneration of muscles in the fourth segment.

There are two possible explanations for the effects produced by the removal of the third abdominal ganglion:

(1) The third ganglion (like the fourth, fifth, and sixth) exerts a preserving influence on the muscles it innervates and its extirpation removes this influence.

(2) The amount of innervation is reduced to below 'subsistence level' for the muscles innervated wholly or partially by that ganglion.

If the first hypothesis is correct it would follow that the first and second ganglia have no preserving influence because the muscles in their segments invariably degenerate. Removal of these ganglia would have no effect.

If the second hypothesis is correct, removal of the second ganglion would cause some degeneration of muscles in the third segment because each ganglion contributes to the innervation of the segment behind its own.

Experiments were carried out with *S. cynthia* and *A. pernyi* to test these hypotheses. The second ganglion was removed from 4 larvae of each species. In every animal except one the longitudinal muscles of the third segment degenerated during metamorphosis. In *S. cynthia* removal of the second ganglion resulted in complete degeneration of the lateral band of the third segment in each animal, complete degeneration of sub-division (c) of the dorsal band in 3 out of the 4 animals, and partial degeneration of sub-division (b) in each animal. Sub-division (a) was of course missing as usual. In *A. pernyi* all 4 animals showed degeneration of the

lateral band of the third segment and two showed degeneration of sub-divisions (b) and (c) of the dorsal band of the third segment. The results differed from those obtained by removing the third ganglion in that muscles of the fourth segment were not affected.

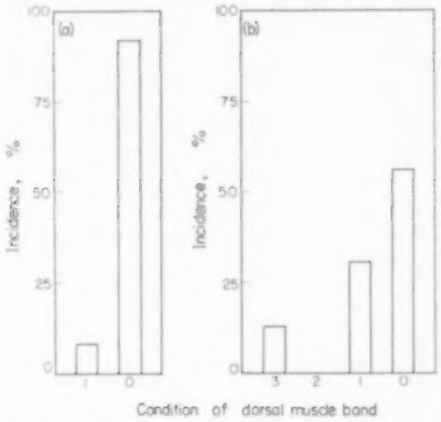

FIG. 4. Histograms showing the effect of removing certain abdominal ganglia of the spinning larva on the lateral muscle band of the pupa of *Samia cynthia*.

Ganglia removed (a) Third, second + third or first + second + third (25 animals).
(b) Second or first + second (17 animals).

4, normal muscle; 0, completely degenerated muscle; other numbers represent degrees of degeneration.

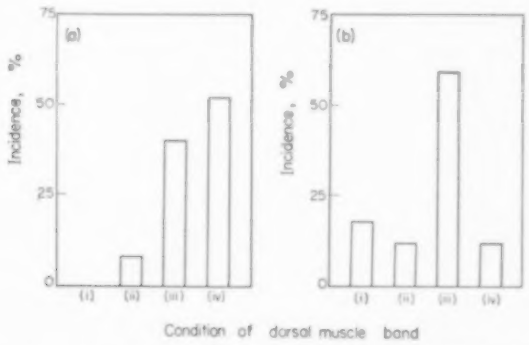

FIG. 5. Histograms showing the effect of removing certain abdominal ganglia of the spinning larva on the dorsal muscle band of the third abdominal segment of the pupa of *Samia cynthia*.

Ganglia removed (a) Third, second + third or first + second + third (25 animals).
(b) Second or first + second (17 animals).

Grouping according to the condition of the sub-divisions (a, b, and c) of the dorsal band on the scale in which 4 represents normal muscle and 0 represents completely degenerated muscle.

Groups:	(i)	(ii)	(iii)	(iv)
	0.4.4	0.2.2	0.3.0	0.1.1
	0.3.3	0.2.1	0.2.0	0.1.0
				0.0.0

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Other experiments were carried out with *S. cynthia* in which the following combinations of abdominal ganglia were removed: first and second (10 survivors); first, second, and third (4 survivors); second and third (5 survivors). The degeneration caused by the extirpation of the second ganglion or the first and second ganglia was rarely as extreme as that caused by the extirpation of the third ganglion alone or in combination with others (Figs. 4 and 5).

The results of these experiments support the hypothesis that the amount of innervation is the critical factor in preserving the muscles from involution. Removal of either the second or the third ganglion leads to the degeneration of muscles in the third abdominal segment. In *A. pernyi* the characteristic degeneration of sub-division (a) of the dorsal band of the fourth abdominal segment is not shown by those animals from which only the second abdominal ganglion has been removed. Apparently the second ganglion does not innervate the fourth segment or if it does the amount of innervation it contributes is too small for its loss to have any effect.

GRADIENTS OF HISTOLYSIS

I have previously described (FINLAYSON, 1956) two gradients that influence the rate of muscle histolysis so that it takes place in two waves, one starting at the anterior end of the body and the other at the posterior end. In the case of the longitudinal muscles of the abdomen these waves stop in the third and seventh segments. The longitudinal muscles of the third segment are never complete in saturniids; at least sub-division (a) of the dorsal band is missing as well as the ventral band. The experiments described above have shown clearly that the muscles in the third segment can easily be induced to degenerate by denervation, but those in the fourth and fifth segments are not affected except in *A. selene* (apart from the special case of sub-division (a) in the dorsal band in *A. pernyi*) and even in this species degeneration of the muscles in these segments is never complete. This is good evidence for the anterior gradient. Similarly, because of the existence of the posterior gradient, it was thought that the muscles of the sixth segment might be more sensitive to denervation than those of the fourth and fifth segments.

A series of experiments were carried out with *S. cynthia* to see if this expectation was justified. From larvae were removed the fourth, fifth, or the sixth abdominal ganglion, or the fourth and fifth ganglia. Only the removal of the sixth had any effect. There was a slight but distinct degeneration of the muscles of the sixth segment in 4 out of 10 animals. There is never any sign of reduction of the longitudinal muscles of the sixth segment in normal *S. cynthia* as there is in the third segment, in which, apart from the usual loss of the ventral band and sub-division (a) of the dorsal band, the remaining muscles are reduced in thickness to about stage 3 in the scale (Fig. 1).

Thus in *S. cynthia* it is possible by ganglionectomy to intensify the normal degeneration in the third abdominal segment and to induce it in the sixth segment but not in the fourth or fifth segments. This evidence supports the theory of two gradients. Additional evidence is available in the results of similar experiments carried out with *A. selene*. Removal of the fifth ganglion or the fourth and fifth

ganglia resulted in degeneration of the muscles of the fifth and sixth segments in 6 out of 10 animals. The degeneration of the muscles in the sixth segment was as extreme or more extreme than in the fifth segment, despite the fact that the sixth ganglion was still intact. Another set of experiments with *A. selene* demonstrated the anterior gradient. The third and fourth ganglia were removed from 13 animals.

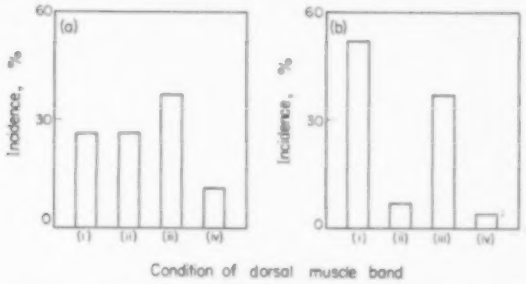

FIG. 6. Histograms showing the effect of removing the third abdominal ganglion ((a) 19 *Antheraea pernyi*, (b) 13 *Actias selene*) or cutting all nerves close to the third abdominal ganglion (14 *Actias selene*) of the spinning larva on the dorsal muscle band of the third abdominal segment of the pupa.

Grouping according to the condition of the sub-divisions (a, b, and c) of the dorsal band on the scale in which 4 represents normal muscle and 0 represents completely degenerated muscle.

Groups: (i) 0.4.4	(ii) 0.4.3	(iii) 0.4.1	(iv) 0.1.1
	0.4.2	0.4.0	0.0.0
	0.3.3	0.3.1	
	0.3.2	0.3.0	
		0.2.2	
		0.2.0	

All showed degeneration of muscles in the third segment, 11 in the fourth segment, and 6 in the fifth segment. The degree of degeneration was progressively less severe from the third to the fifth segment. In none of the animals was there any degeneration of muscles in the sixth segment. In these experiments loss of innervation from the fourth ganglion leads to degeneration in the fourth and fifth segments and loss of innervation from the fifth segment leads to degeneration in the fifth and sixth segments. The important difference in the results is that the degeneration induced in the fifth segment by removing the fourth ganglion is less severe than that induced in the sixth segment by removing the fifth ganglion. Experiments in which the third ganglion only was removed have already been described. In only a few animals was there a very slight degeneration of the muscles of the fourth segment. The muscles of the sixth segment are, therefore, more sensitive to denervation than those of the fourth or fifth segments.

EFFECT OF SEVERING THE MEDIAN NERVE OR THE NERVE CORD

The details of the innervation of the muscles are not known, but an obvious contribution to the innervation of any segment is provided by the median nerve of the ganglion in the preceding segment. It was possible that the similarity in

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results obtained by removing either the second or the third ganglion might be explained by the fact that both operations sever the median nerve of the second ganglion. Experiments were therefore carried out in which the median nerve of the second ganglion was severed in 6 *S. cynthia* and 4 *A. pernyi*. In 5 of the *S. cynthia* there was abnormal degeneration in the third segment but all the *A. pernyi* were normal.

By simply severing the whole nerve cord plus median nerve between the second and third ganglion in *S. cynthia* the same effect was obtained (5 animals). This is a simpler operation and it was assumed that the effect was being produced by severing the median nerve. However, when the same operation was tried on *A. pernyi* there followed distinct degeneration of muscles of the third segment (3 animals).

If the median nerve of the second ganglion alone is responsible for the preservation of the persisting muscles in the third segment, then severing the other nerves of the third ganglion would have no effect. That this is not true was demonstrated by severing all the nerves from the third ganglion and leaving intact the median from the second and its branches. In *A. pernyi* 7 animals out of 11 showed degeneration of muscles in the third segment after this operation and although only 2 *S. cynthia* were tried, both showed degeneration.

It has been shown, therefore, that a similar reduction of segmental muscles in the third abdominal segment can be obtained by the following different operations:

(1) Removal of the second abdominal ganglion.

(2) Removal of the third abdominal ganglion.

(3) Severance of the median nerve of the second abdominal ganglion (*S. cynthia* only).

(4) Severance of the nerve cord and median nerve between the second and third abdominal ganglia.

(5) Severance of the nerves of the third abdominal ganglion leaving intact the median of the second abdominal ganglion and its branches.

These results indicate that no one nerve or set of nerves is exclusively involved in the preservation of the muscles; much more likely is the explanation that a critical amount of innervation from any source is sufficient to maintain the muscles in a normal condition.

SEQUENCE OF MUSCLE DEGENERATION IN THE DORSAL BAND

A comparative study of the normal muscle pattern of the pupa and the results of numerous experiments have shown that the three sub-divisions of the dorsal band break down in a regular sequence. The first to degenerate is (a), as shown by controls of *A. pernyi*, *A. selene*, and *S. cynthia* (Fig. 1) and experiments on *A. pernyi* (Fig. 2). Next to degenerate is (c), as shown by experiments with *A. pernyi*, *A. selene*, and *S. cynthia* and normal pupae of *A. polyphemus*. Finally the middle sub-division (b) degenerates.

DISCUSSION

This investigation has been successful in so far as it has demonstrated that differences like those between *Galleria mellonella* and the two saturniids *Antheraea pernyi* and *Hyalophora cecropia* also exist between various species of saturniids. *Actias selene* is similar to *Galleria mellonella* in its response; denervation in the larva leads to rapid degeneration of normally persisting muscles of the pupa in all four segments of the abdomen in which these muscles occur. *A. selene*, like *G. mellonella*, does not have a pupal diapause, but no obvious correlation such as I suggested (FINLAYSON, 1956) between diapause and persistence of muscles after denervation could be found. The strain of *S. cynthia* used was a non-diapausing one and non-diapausing *A. pernyi* were produced by rearing the larvae in a long photoperiod. Neither of these stocks responded like *G. mellonella* or *A. selene*.

Attention was concentrated on the muscles of the third abdominal segment when it was discovered that the pattern of persisting pupal muscles in that segment was constant in *A. pernyi*, *A. selene*, and *S. cynthia*. Denervation of these muscles in the fully-grown larva causes them to degenerate during pupation. Similar results were obtained by a variety of operations that interfered with the innervation. It is clear that there is no specific trophic nerve or nerves but that reduction in the amount of innervation causes degeneration during metamorphosis. These findings are in line with results obtained in experiments on the influences of innervation in regeneration in Amphibia (SINGER, 1954). It was also established that the second abdominal ganglion exerts a preserving influence on the muscles of the third abdominal segment. This result is contrary to the suggestion made previously (FINLAYSON, 1956) that the muscles of the third segment were under the preserving influence of the third ganglion only.

A striking difference between *A. pernyi* and the other two species is shown in the response to removal of the third abdominal ganglion. In *A. pernyi* sub-division (a) of the dorsal band of the fourth segment degenerates. This never happens in the other two species. It is significant that this is the sub-division that is always lacking in the third segment of the pupa of all species of saturniid studied so far. There is in fact a sequence of degeneration within the dorsal band. Sub-division (a) is the first to degenerate, then (c) and finally (b). Therefore, by removing the third abdominal ganglion of *A. pernyi* it is possible to produce in the fourth abdominal segment the pattern of persisting muscles in the dorsal band that is characteristic for the third segment of normal pupae.

The sequence of events during degeneration of the dorsal band seems obviously to be a reflection of the pattern of innervation and lends further support to the hypothesis that the nervous system plays a positive role in the determination of the pattern of the persisting longitudinal muscles (FINLAYSON, 1956).

The multiple nature of the innervation in the lateral band is shown particularly well by the results of removal of the third abdominal ganglion or the severing of all nerves from the third ganglion (including the median of the second ganglion) in *A. selene* (Fig. 3). Group X consists of the animals that have several normal

fibres in an otherwise completely degenerated lateral band. Presumably they have been maintained by innervation from the second abdominal ganglion.

That the effects of denervation depend on the gradients of histolysis (FINLAYSON, 1956) has been confirmed. In *S. cynthia*, the species least affected by denervation, degeneration can be induced in the third and sixth segments, in *A. pernyi* in the third, fourth, and sixth segments, and in *A. selene* in all four segments. In this short-term degeneration of muscles that occurs at metamorphosis, as opposed to long-term atrophy (FINLAYSON, 1956; ROEDER and WEIANT, 1949; NOVOTNÝ, 1958), the lack of or reduction in amount of innervation can only result in degeneration of the muscles if the wave of histolytic influence has reached the denervated muscle. There are inter-specific differences in the rate or extent of travel of these waves, hence the differences in response obtained with different species. When the next phase in development becomes dominant, whether it be the onset of diapause or the development of the adult, the histolytic phase has finished as far as these muscles are concerned, and denervation has no effect on them.

It is clear that the effects of denervation on the maintenance of muscles in the pupa depends on the interaction of the endocrine balance of the blood, the gradients of histolysis, and the loss of innervation.

In studies on the degeneration of flight muscles in aphids JOHNSON (1959) found that decapitation prevented muscle breakdown. He also found that the thoracic muscles of decapitated aphids broke down when they were joined in parabiosis with intact aphids. It is possible, as JOHNSON suggests, that removal of the ganglia in the head may interfere with the neural influence on the flight muscles so that they will break down in the appropriate hormonal environment.

WIGGLESWORTH (1956) found that denervation of the intersegmental muscles of *Rhodnius prolixus* did not affect the cycle of growth and involution that these muscles undergo. Denervation produces no obvious degeneration in the muscles of the larva of *Galleria mellonella* (FINLAYSON, 1956) or *Lymantria dispar* (KOPEĆ, 1923), and very little in *Periplaneta americana* (ROEDER and WEIANT, 1949; NOVOTNÝ, 1958). The loss of innervation in *Rhodnius* would not be expected to interfere with the involution of the muscles but their subsequent growth is more surprising. However, the integumental muscles of saturniid moths also develop in the absence of central innervation (FINLAYSON, 1956). There can be no doubt of the importance of innervation in growth of insect muscles in certain cases (KOPEĆ, 1923; NÜESCH, 1952; SUSTER, 1933), but the results described in the present paper show that innervation is but one of the factors influencing the maintenance of muscles.

The mode of action of the innervation is not known at present, but it is possible, as WIGGLESWORTH (1955) and FRASER (1959) have suggested, that the neurosecretory cells of the abdominal ganglia are concerned in this trophic function of the nervous system. Additional support for this supposition is the fact that removal of all the thoracic ganglia (NÜESCH, 1956) or all the abdominal ganglia (FINLAYSON, 1956) from pupae of *Antheraea polyphemus* has apparently no effect on development

other than to prevent the growth of the major muscles. It is planned to test this hypothesis by destroying the neurosecretory cells of appropriate abdominal ganglia, now that the responses of muscles to denervation are known in several large Lepidoptera.

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THE RESPIRATORY SYSTEM OF THE EGG-SHELL OF *CALLIPHORA ERYTHROCEPHALA*

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Abstract—It is shown that *Calliphora* eggs require an external source of oxygen during the incubation period, and that they die in de-aerated water but develop equally well in either aerated shallow water or moist air. When immersed in water the film of air held in the outer meshwork layer of the median area between the hatching lines functions as a plastron. If the plastron is completely blocked with various non-toxic substances the eggs die. If the plastron is partially blocked, the normal time for development is increased. Replacing the air in the cavities between the vertical columns of the chorion by aerated water results in a much increased incubation period.

The structure of the respiratory system of the egg-shell of *Calliphora* has been re-examined using the techniques adopted by WIGGLESWORTH and BEAMENT and HINTON. A thick middle layer exists between the inner and outer meshwork layers of the median area, and aeropyles through the middle layer connect the air films on either side. The inner layer of the median area extends dorsally into each hatching fold, and there connects by an open network with the inner meshwork elsewhere. Apart from this open network in the plane of each hatching line, the inner sheet elsewhere is continuous, and air is confined to the spaces between the vertical columns as described by HINTON.

INTRODUCTION

RECENTLY the plastron of a large number of eggs has been described (HINTON, 1959,1960a, b, c). The structure of the egg-shell of *Calliphora erythrocephala* is shown in Fig. 1. Except for the median area between the hatching lines, the chorion consists of a thin inner layer connected by vertical columns to a somewhat thicker outer layer, which in turn is covered by a thin coat of cement (HINTON, 1960b). The chorion of the median area between the hatching lines is in three layers. The thin inner sheet is continuous and connected by hexagonally arranged short vertical columns to a thick middle layer. The continuous air film of the inner meshwork layer is connected with a similar air film in the outer meshwork layer by a large number of holes or aeropyles which penetrate the middle layer. Struts arise vertically from the latter and widen, branch, and often anastomose apically to form '. . . a fine and open hydrofuge network at the surface of the median area' (HINTON, 1960b). In the plane of each hatching line the inner layer is no longer continuous but is replaced by an open network. Thus air enters the egg through the outer meshwork layer and passes directly into the inner meshwork layer of the chorion. If the egg is covered by water, as may frequently occur when it rains, the outer meshwork of the median area forms the water–air interface of the plastron.

Although some of the respiratory system of the egg-shell of *Calliphora* is agreed upon, a dispute has arisen over certain important functional and structural details. The claim by WIGGLESWORTH and BEAMENT (1950, 1960) that the spaces between the vertical columns are filled with water and not air, and that the air is confined to the substance of the columns and the inner layer, has not been

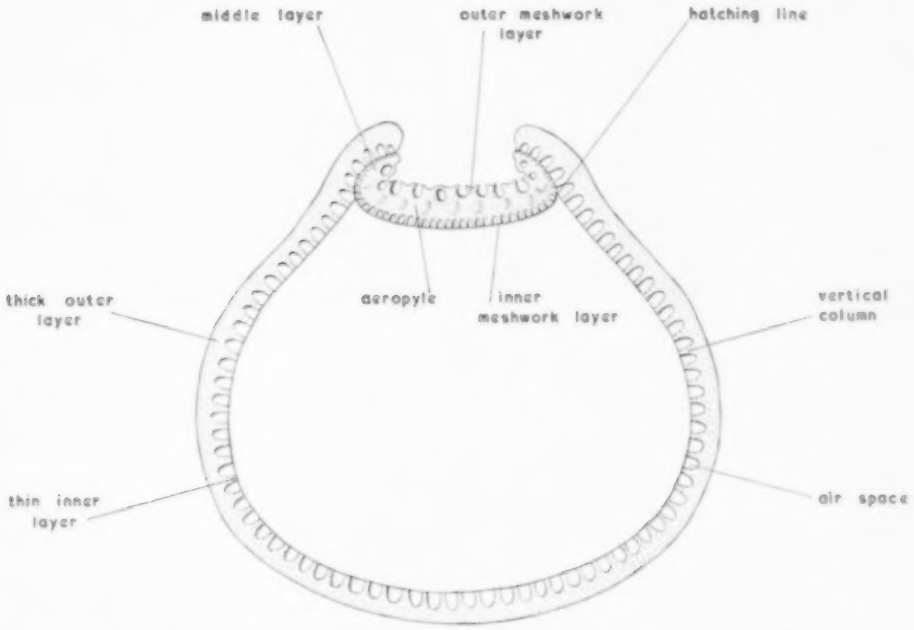

FIG. 1. *Calliphora erythrocephala*. Diagrammatic transverse section through the chorion (based on HINTON, 1960c)

substantiated. They also consider that the inner layer is not continuous but is spongy. Moreover, they do not mention a middle layer to the median area, and suggest that the cavities between the vertical columns may be concerned in water conservation.

In this paper the effect of blocking the plastron with various substances in order to demonstrate its respiratory function is described. The details of the egg-shell of *Calliphora*, which are in dispute, have been elucidated by experiment and are discussed.

THE INCUBATION PERIOD

All experiments were caried out on live *Calliphora* eggs obtained as soon as possible after they were laid. Moreover, only eggs laid at the same time were used for the control and experiments in each series. This ensured that variations in the

incubation period relative to the controls were insignificant. Nevertheless, an incubation period histogram (Fig. 2) has been constructed from Table 1 for comparison. Table 1 shows that the first eggs hatched 49 hr and the last 67 hr after

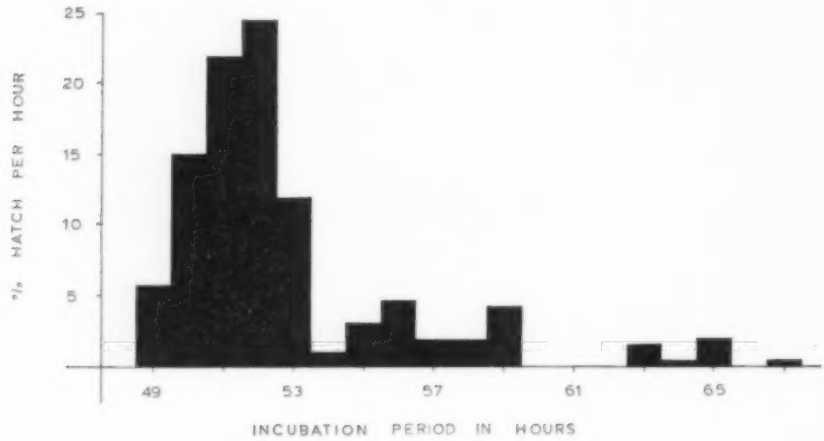

FIG. 2. *Calliphora erythrocephala*. Incubation period histogram.

TABLE 1—INCUBATION PERIOD OF 200 EGGS OF *Calliphora erythrocephala* (19–21°C)

Mean incubation period (hr)	Total number of eggs hatched	Per cent hatch per hour
48	0	0
49	11	5·7
50	40	15·0
51	82	21·8
52	129	24·4
53	152	11·9
54	154	1·0
55	160	3·1
56	169	4·7
57	173	2·0
58	177	2·0
59	184	3·7
60	184	0
61	184	0
62	184	0
63	187	1·6
64	188	0·5
65	192	2·0
66	192	0
67	193	0·5

being laid. Out of 200 eggs, 193 or 96·5 per cent hatched and 78·8 per cent of these were in the peak period from 49 to 53 hr.

Eggs were immersed to a depth of ½–1 cm in either aerated or de-aerated tap water, and controls were placed on moist filter paper. The hatch of about 95 per

TABLE 2—EFFECTS OF IMMERSING *Calliphora* EGGS IN AERATED AND DE-AERATED SHALLOW WATER

Batch	No. of eggs in aerated water	Hours to hatch	Number hatched	No. not hatched	Per cent hatch
1	5	37	5	0	100
2	5	44	4	1	80
3	20	40	20	0	100
4	30	46	29	1	96·6
5	30	51	27	3	90
6	30	48	30	0	100
Total	120	51	115	5	95·8

Batch	No. of eggs in de-aerated water	Hours to hatch	Number hatched	No. not hatched	Per cent hatch
1	30	—	0	30	0
2	30	—	0	30	0
3	30	—	0	30	0
4	30	—	0	30	0
Total	120	—	0	120	0

Batch	No. of eggs in moist air (control)	Hours to hatch	Number hatched	No. not hatched	Per cent hatch
1	5	34	5	0	100
2	5	42	5	0	100
3	20	40	20	0	100
4	30	44	30	0	100
5	30	44	25	5	83·3
6	30	48	28	2	93·3
Total	120	48	113	7	94·1

cent (Table 2) in both the aerated experiment and the control shows that *Calliphora* eggs will hatch in shallow water, and that the incubation period is not appreciably affected by immersion. However, if the depth of water covering the eggs is increased the development of the eggs is delayed. It is suggested that this delay is due to lack of oxygen.

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THE ROUTE OF ENTRY OF AIR INTO THE EGG

The need for an external supply of oxygen was shown by placing thirty eggs in de-aerated water exposed to de-oxygenated air. In the control there was a 100 per cent hatch of thirty eggs in 48 hr, but none of the eggs that had no external source of oxygen survived. Similarly, when thirty eggs were placed in de-oxygenated air of 100 per cent humidity none hatched.

TABLE 3—EFFECTS OF COMPLETELY BLOCKING THE PLASTRON OF THE EGG OF *Calliphora*
(Figures in brackets refer to the control eggs)

Batch	Seal used	Number of eggs	Hours to hatch	Number hatched	No. not hatched	Per cent hatch
1	Vaseline	95	67	4	91	4·2
		(95)	(56)	(92)	(3)	(98·9)
2	Grease	165	76	9	156	5·5
		(165)	(67)	(156)	(9)	(94·5)
3	Whitehead's varnish	75	—	0	75	0
		(75)	(66)	(70)	(5)	(93·3)
4	Paraffin wax	90	—	0	90	0
		(90)	(56)	(87)	(3)	(96·6)

Table 3 gives the results of blocking the plastron with various substances. When it is completely blocked, almost 100 per cent of the eggs die, and the time required for development in the few that survive the treatment is increased. These results confirm previous observations by demonstrating (1) that the eggs do not contain enough air to complete their development, and (2) that the route of entry of the air is via the median area. Partial blockage of the plastron (Table 4) increases

TABLE 4—EFFECTS OF PARTIALLY BLOCKING THE PLASTRON OF THE EGG OF *Calliphora*

Batch	Seal used	Number of eggs	Hours to hatch	Number hatched	No. not hatched	Per cent hatch
1	Vaseline	60	60	60	0	100
2	Grease	60	64	56	4	93·3
3	Varnish	60	64	2	58	3·3
4	Wax	60	82	42	18	70
5	None (control)	60	50	60	0	100

the incubation period by as much as 10 hr. Table 4 also shows that Whitehead's varnish is toxic to *Calliphora* eggs. This was further confirmed by covering parts of eggs with the varnish, and exposing others to the vapour. Almost none survived the treatment.

Air in the eggs was replaced by immersion in 100 per cent alcohol for a few seconds, and then immediately replaced again by either aerated or de-aerated water. Control eggs were subjected to the same process, desiccated, and then placed in

aerated shallow water. The eggs suffered no ill effects from their brief immersion in alcohol during which air could be seen bubbling from the median area of each egg. The results (Table 5) show that (1) eggs flooded with aerated water will

TABLE 5—EFFECTS OF FLOODING *Calliphora* EGGS WITH AERATED OR DE-AERATED WATER

Batch	Number of eggs	Hours to hatch	Number hatched	No. not hatched	Per cent hatch
(a) Control eggs in aerated shallow water					
1	15	48	15	0	100
2	30	56	30	0	100
3	30	58	27	3	90
4	30	40	30	0	100
5	30	48	26	4	86·6
Total	135	58	128	7	94·8
(b) Eggs flooded with aerated water					
1	15	82	14	1	93·3
2	30	87	19		
		150	28	2	93·3
3	30	87	16		
		150	26	4	86·6
4	30	60	11		
		82	14		
		150	20	10	66·6
5	30	68	16		
		80	20		
		150	27	3	90
Total	135	150	115	20	85·1
(c) Eggs flooded with de-aerated water					
1	15	—	0	15	0
2	30	—	0	30	0
3	30	—	0	30	0
4	30	—	0	30	0
5	30	—	0	30	0
Total	135	—	0	135	0

develop but are subject to a delay in the incubation period, and (2) eggs flooded with de-aerated water will not hatch. It is concluded that the plastron is respiratory in function.

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DISCUSSION

There has been no previous attempt to demonstrate experimentally that the plastron of *Calliphora* eggs has a respiratory function. It is shown here that such eggs do not contain sufficient air for their development, and that they utilize oxygen from either the surrounding air, or, if immersed, from the ambient water. Moreover, the eggs develop equally well when immersed in shallow aerated water or when exposed to moist air. Although not explicitly stated in previous work, the inference has been that oxygen enters into the air spaces of the shell through the median area, since it is only there that entry can be effected without having to diffuse through a solid and comparatively thick chorion layer. This view is here corroborated.

These findings confirm HINTON's conclusion (1960b) that '. . . the film of air held in the outer layer of the area between the hatching lines functions as a plastron when it rains or the egg is otherwise immersed in water'. Such immersion is a normal environmental hazard, and it was found that eggs will hatch when continuously immersed under 12 in. of aerated water, although this treatment has the effect of considerably lengthening the incubation period. Furthermore, HINTON (*op. cit.*) has demonstrated experimentally that the plastron, which is held in position by an open hydrofuge network, is capable of resisting wetting by hydrostatic pressures far greater than any to which it is likely to be exposed in nature. This he explains by showing that the surface tension of the liquids on decomposing organic matter in which the eggs are laid is much lowered by surface active substances. He has shown that the resistance of the plastron to hydrostatic pressure is directly proportional to the surface tension of the liquid.

WIGGLESWORTH and BEAMENT (1950), in describing the structure of the eggshell of *Calliphora*, state that the spaces between the vertical columns are filled with a glassy material which is 'mainly water'. Further, they suggest (*loc. cit.*, p. 447) that '. . . aeration or "pneumatization" of the cavities in the chorion does not seem to take place in the normal *Calliphora* egg', and that '. . . air is confined to the porous innermost layer of the chorion and to the substance of the vertical columns'. They base their conclusions upon experiments with oil droplets and cobalt sulphide. HINTON (*op. cit.*) disagrees with their findings, stating that in large numbers of *Calliphora* and *Lucilia* eggs, observation and dissection showed that the chorion was always air-filled when the egg left the ovipositor. He believes that the innermost layer of the chorion is continuous, and that the air is confined to the spaces between the vertical columns, as in *Syrphid* eggs. These statements can now be re-examined in the light of new experimental evidence in order to elucidate the dispute which has arisen.

Aeration of the cavities was confirmed by dissection of several eggs shortly after oviposition. Paraffin oil was applied to the outer layer of the median area and could be seen displacing air in the outer meshwork, running through the aeropyles and displacing air between the vertical columns. Providing sufficient oil was applied, it overflowed from the median area and ran through the inner layer, which is here an open network, into the air-filled spaces between the vertical

columns in other parts of the chorion. Paraffin oil when applied in very small quantities to the inner surface of the inner layer of the chorion did not penetrate and displace air between the columns. Great care is necessary in this process otherwise a false impression is obtained. Moreover, the curved chorion easily cracks when flattened, thus allowing oil to penetrate. There are, however, small scattered irregular patches which do not occupy the same positions in homologous eggs, where the oil will penetrate. But most of the thin inner layer is impervious to paraffin oil, confirming HINTON's (*op. cit.*) observations and conclusion. In *Calliphora* eggs, therefore, the inner layer of chorion is continuous, except in the hatching folds, and not open as it is in Muscinae (HINTON, 1960c).

Using oil droplets also reveals the presence of a middle layer to the median area. This was completely overlooked by WIGGLESWORTH and BEAMENT, and was first reported in detail by HINTON (*op. cit.*). It connects the outer meshwork via aeropyles with the inner meshwork layer of the chorion (Fig. 1). Not only is it possible to distinguish this layer in optical section, but, using WIGGLESWORTH's latest and rather lengthy osmium tetroxide–ethyl gallate technique (1959), it was possible in sections 1 μ thick to confirm the existence of the middle layer, and to show that the inner layer is no longer continuous in the plane of each hatching line but is replaced by an open network.

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WIGGLESWORTH and BEAMENT (*op. cit.*) first examined the structure of the egg-shell of *Calliphora* using a cobalt sulphide technique. The validity of their results was questioned by HINTON (1959) when he stated that in lightly injected eggs the sulphide adheres to the vertical columns giving the appearance of having actually impregnated the columns, as thought by WIGGLESWORTH and BEAMENT. When heavily injected the spaces between the columns are seen to be filled with sulphide, indicating air to be present between the vertical columns and not in them. However, in their recent paper, WIGGLESWORTH and BEAMENT (1960) admit that 'the cobalt sulphide technique is not wholly satisfactory because it is apt to form an irregular granular precipitate'. Their improved method consists in filling the respiratory system of the egg with a mixture of equal parts olive oil and kerosene, fixing with osmium tetroxide and colouring with ethyl gallate. This work has been repeated.

Thin sections were cut by WIGGLESWORTH's method (1959), and in addition whole pieces of the chorion were treated and mounted. The osmium–ethyl gallate method undoubtedly shows with great clarity the hexagonal pattern of air canals, but these are visible in unstained preparations in any case. It also confirms the presence of a middle layer to the median area as already mentioned. Beyond that it is very doubtful whether the method does in fact show the existence of air in the inner chorionic layer and vertical columns, together with water in the spaces between the columns, as WIGGLESWORTH and BEAMENT suppose. Ethyl gallate will adhere to and apparently penetrate the substance of the chorion if the eggs are left in it sufficiently long, but this does not prove the presence of an 'aeroscopic sponge'. As HINTON (1960b) says, whereas '. . . the air spaces in insect egg-shells are considerably wider than the mean free path of the oxygen

molecule, . . . when the columns or struts supporting the air spaces are examined with the electron microscope, it immediately becomes clear that they are not "spongy" at the necessary level'.

Another feature of the osmium–ethyl gallate technique is that where the vertical columns are coloured it appears to be the outermost layer which is most deeply stained, as also noted by WIGGLESWORTH and BEAMENT. This suggests that penetration is not extensive. They also admit that a possibility for error in their observations is that the oil was inaccessible to the reagents used. An oil and kerosene mixture was subjected to the osmium–ethyl gallate process in a test tube, and became coloured a deep blue black. It was found that this mixture readily and firmly adheres to a smooth damp glass surface, but adhesion decreases and cohesion increases as the surface becomes drier. There is no reason to suppose that similar adhesion to the surface of the vertical columns and the inner layer does not take place in the damp chorion, so that at first sight the columns themselves and the inner layer appear to be impregnated. Consequently, when WIGGLESWORTH and BEAMENT injected and treated desiccated eggs it is not surprising that they found the blue-black mixture filling a part of the space between the vertical columns, since the mixture was no longer adhering to the columns to the same extent. Judging from these results the osmium–ethyl gallate technique when applied to the respiratory system of the egg of *Calliphora* seems to be as inconclusive and inappropriate as the cobalt sulphide technique.

The essential respiratory feature in the egg-shell of the blowfly *Calliphora* is therefore a continuous film of air held between the vertical columns in the inner part of the chorion. It connects with another film of air held in the outer meshwork layer of the median area between the hatching lines, and this functions as a plastron when the egg is immersed in water.

Acknowledgement—I am indebted to Dr. H. E. HINTON for suggesting the problem.

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DEVELOPMENT OF THE CHOLINERGIC SYSTEM IN INSECT EGGS*

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Abstract—Precise evidence has been obtained for the presence of acetylcholine, acetylcholinesterase, and choline acetylase in developing eggs of *Musca domestica* (L.) and *Oncopeltus fasciatus* (Dall.). During embryogenesis choline acetylase appears first; acetylcholine was detected only after the appearance of acetylcholinesterase which appears late in development. The amounts of substrate and enzymes increase progressively with the development of embryo until the time of hatching.

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INTRODUCTION

THE evidence has been accumulating in recent years that the essential elements of the cholinergic system, viz. acetylcholine (ACh), cholinesterase (ChE), and choline acetylase (ChA), are present in the central nervous system of insects (see reviews by SMALLMAN, 1958; ROEDER, 1958). The functional significance of these cholinergic elements in nervous activity of adult insects has been discussed (see reviews by COLHOUN, 1959a, b). However, in view of the uncertainty surrounding the role of ACh in nervous activity of adult insects, it was felt that a knowledge regarding the occurrence and time of appearance of such a system in relation to embryogenesis might provide additional information in this matter.

Although the occurrence of ChE in insect eggs has been known for some time (see review by SMITH and WAGENKNECHT, 1959), it is only recently that the occurrence of an ACh-like substance along with ChE and ChA has been shown in the eggs of *Chilo suppressalis*, *Barathra brassicae* (YUSHIMA, 1958), and *Bombyx mori* (CHINO, 1957). Moreover, as yet no specific correlation is possible between the appearance of these cholinergic elements and the embryonic development.

The present work, therefore, attempts to establish the time of appearance and occurrence of ACh, ChE, and ChA during embryonic development of the house fly (*Musca domestica* L.) and milkweed bug (*Oncopeltus fasciatus* Dall.).

MATERIALS AND METHODS

Eggs of the house fly, *Musca domestica*, were collected from a laboratory colony according to the method of FISHER and JURSIC (1958). The age of the eggs used was controlled by offering the oviposition dishes for only 1 hr. The eggs were then washed from the oviposition dishes into a beaker, the water decanted,

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and the eggs collected. Increased oviposition of eggs was obtained by adding a few drops of 5 per cent ammonium carbonate solution to the oviposition dishes. Eggs of the milkweed bug were collected from a laboratory colony of adults. Large numbers of eggs were collected by exposing rolled plastic wire screens to females to oviposit for 24 hr. After 24 hr the screens were removed from the cages, and the eggs removed with a camel hair brush. Eggs were incubated at 28°C until used for ACh assay or for determining the enzymic activity. The incubation period under these conditions was approximately 12 hr for the house fly and 5½ days for the milkweed bug.

Extraction, Characterization, and Estimation of Acetylcholine

(a) *Biological assay*

The method used for the extraction of ACh from the eggs was essentially that of LEWIS and SMALLMAN (1956) for adult insect heads. The eggs of the house fly and the milkweed bug were collected at chosen intervals, weighed, and immediately immersed from 1 to 2 min in 5 ml of boiling HCl-acidified frog Ringer's solution (NaCl 7·0 g; KCl 0·14 g; $CaCl_2$ 0·12 g; in 1 litre of distilled water, acidified to pH 4·0 with 0·3 N HCl). On cooling, 0·5 ml of 10 per cent trichloroacetic acid (TCA) was added, and the eggs were homogenized in a Potter–Elvehjem glass homogenizer. The homogenate was decanted and centrifuged for 5 min at 4000 rev/min and the supernatant collected. The residue was resuspended in HCl-acidified Ringer's solution, homogenized, centrifuged, and the supernatant collected. The supernatants were pooled and the pH adjusted to 6·0 with 0·3 N NaOH. During the addition of alkali, care was taken to stir the extract continuously to avoid the loss of ACh (COLHOUN, 1958a). The volume of extract was adjusted to 10 ml by the addition of unbuffered frog Ringer's solution and divided into two aliquots of 5·0 ml each. One aliquot was termed 'test extract' and the other 'blank extract'. The 'blank' was brought to pH 11·5 by the addition of 0·3 N NaOH and momentarily heated to destroy ACh, then cooled and neutralized by the addition of 0·3 N HCl. Small aliquots of these 'test' and 'blank' extracts were used in 5 ml of $NaHCO_3$ buffered frog Ringer's solution (NaCl 7·0 g; KCl 0·14 g; $CaCl_2$ 0·12 g and $NaHCO_3$ 0·2 g, in 1 litre of distilled water) for the estimation of ACh. Usually 1 g of eggs was utilized in each assay except in the late stages of development when only 200 mg of eggs were used.

The estimation of ACh in the 'test' extract was carried out by the use of the frog's rectus abdominis muscle, corrected for possible sensitizing agents according to FELDBERG (1945). The time allowed for the contraction of the muscle was 1½ min and the interval allowed between contractions was 1½–3 min.

(b) *Chemical identification of acetylcholine*

The ACh present in eggs of the house fly and the milkweed bug was also identified by paper chromatography and by the use of the chemical test of HESTRIN (1949). Only 11-hr-old eggs of the house fly and 5-day-old eggs of the milkweed bug were used for this study.

One to two grammes of eggs were homogenized in 10 per cent TCA and then centrifuged at 4000 rev/min for 5 min. The supernatant was decanted and the residue was suspended in distilled water, homogenized, centrifuged, and supernatant collected. The supernatants were pooled and the TCA removed by shaking the extract four times with an equal volume of ether saturated with water. The extract was then reduced to dryness *in vacuo*. The residue was dissolved in 1 or 2 ml of distilled water and used either for paper chromatography or for chemical assay of ACh by the technique of HESTRIN (1949).

(i) *Characterization of acetylcholine by paper chromatography*. The ascending method of WILLIAMS and KIRBY (1948) was followed for the identification of ACh. It has been suggested that the anions associated with ACh influence the R_f of ACh during chromatography (WHITTAKER and WIJESUNDERA, 1952; CHEFURKA and SMALLMAN, 1956). In the present study it was observed that the anions not only affected the R_f, but that also it was possible to separate the different anionic forms of ACh by paper chromatography. Therefore, for reference it become necessary to chromatograph the same anionic form of ACh as expected in the extract.

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The extracts were placed upon Whatman No. 1 filter paper by the use of a micropipette; for comparison, ACh subjected to the same chemical treatment as the extract was also chromatographed. The following solvents were used successfully to chromatograph ACh:

(a) Butanol–acetic acid–water; 4 : 1 : 5 (PARTRIDGE, 1948).
(b) Butanol saturated with water (WHITTAKER and WIJSUNDRA, 1952).
(c) Butanol–ethanol–acetic acid–water; 8 : 2 : 1 : 3 (AUGUSTINSSON and GRAHN, 1953).

After application of extract and standard ACh to the paper, the chromatograms were run for 16 hr. They were then removed and dried in an aerated oven at 110°F, and the choline esters were located as blue spots by spraying the chromatograms with phosphomolybdic acid, followed by reduction with acidic stannous chloride (CHARGAFF *et al.*, 1948).

(ii) *Chemical characterization*. After drying *in vacuo*, the residue was dissolved in 1 ml of distilled water and treated according to the method outlined by HESTRIN (1949). The absorption spectrum was determined by using the Beckman spectrophotometer, Model DU. The amounts of ACh in the residue were determined by comparison with a calibration curve obtained with acetylcholine bromide.

Cholinesterase

The cholinesterase activity was measured by the standard manometric technique. Eggs at different stages of development were weighed and homogenized in Augustinsson's Salt Solution (AUGUSTINSSON, 1948), using a Potter–Elvehjem glass homogenizer. The conditions for assay were 0·5 ml homogenate, containing 250 mg eggs/ml; 2·0 ml $NaHCO_3$ (0·025 M final); 0·2 ml acetylcholine bromide (0·01 M final); final pH was 7·4, temperature 37·5°C, and the gaseous phase 5 per cent CO_2 and 95 per cent N_2. All determinations were made in duplicate and

corrected for non-specific hydrolysis of substrate, and activities were calculated as b_{30}'s (μl of CO_2 evolved by 125 mg eggs/30 min) by the method of ALDRIDGE *et al.* (1949).

For characterization of the types of ChE present, the substrates ACh bromide, butyrylcholine (BuCh), benzoyl choline (BzCh), and acetyl-β-methyl choline (AMeCh) were used at various concentrations.

Choline Acetylase

The ChA activity was measured by incubating homogenates of eggs for 15 min at 25°C with the following (μM/ml): cysteine, 8·0 (pH 6·8); KCl, 26·5; Mg Cl_2, 5·0; acetyl phosphate, 15·0; choline chloride, 15·0; CoA, 0·1; eserine sulphate, 0·39; phosphate buffer (pH 6·8), 6·7; phosphotransacetylase obtained from *Clostridium kluverii*, 0·28 mg protein/ml. The quantity of eggs in the final reaction mixture was 5 mg of eggs/ml. Homogenates of eggs at stages of development were prepared in 0·9 per cent KCl-cysteine. The reaction was stopped at the end by adding sufficient 0·3 N HCl to bring the pH of the reaction mixture to 4·0.

The ACh formed in the mixture was determined by both the frog's rectus abdominis muscle and the chemical method of HESTRIN (1949). When the technique of HESTRIN was used the incubate was first maintained at 100°C for 4 min at pH 4·0 to destroy acetyl phosphate in the incubation mixture before performing the test. This was necessary because acetyl phosphate also gives the same colour reaction as ACh. This method for assaying ChA activity has been utilized successfully by COLHOUN (1958b) for tissue homogenates of the roach and house fly.

TABLE 1—COMPARATIVE AMOUNTS OF ACETYLCHOLINE PRESENT IN THE EGGS OF THE HOUSE FLY AND MILKWEED BUG DURING DIFFERENT PERIODS OF DEVELOPMENT (DETERMINED BY THE USE OF FROG'S RECTUS ABDOMINIS MUSCLE)

Species	Age of eggs	ACh (μg/g)
House fly	1–3–5–7 hr	Nil
	9 hr	4·6 ± 0·48*
	11 hr	488·0 ± 22·8
Milkweed bug	1–2–3 day	Nil
	4 day	4·1 ± 0·32
	5 day	201·0 ± 13·8

* Standard deviation of the mean.

RESULTS

Acetylcholine

The ACh content of the house fly and milkweed bug at different stages of embryogenesis is given in Table 1. These results were determined by use of the frog's rectus abdominis muscle. No ACh was detectable during early stages of

development and appears first at 9 hr in eggs of the house fly and 4 days in the milkweed bug. However, just before hatching, high amounts of ACh were present at 11 hr in the house fly and at 5 days in the eggs of the milkweed bug.

TABLE 2—COMPARATIVE AMOUNTS OF ACETYLCHOLINE PRESENT DURING LATER STAGES OF DEVELOPMENT IN THE EGGS OF THE HOUSE FLY AND MILKWEED BUG (AS DETERMINED BY THE CHEMICAL METHOD OF HESTRIN, 1949)

Experiment	ACh (μg/g)	
	House fly	Milkweed bug
1	450	160
2	467	170
3	470	165
Average	462	165

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Eggs of these two species were then assayed by the chemical test of HESTRIN (1949), using 5-day-old eggs of the milkweed bug and 11-hr-old eggs of the house fly. The results given in Table 2 show that ACh was present in the eggs in amounts determined by the use of the frog's rectus abdominis muscle. Fig. 1 shows that

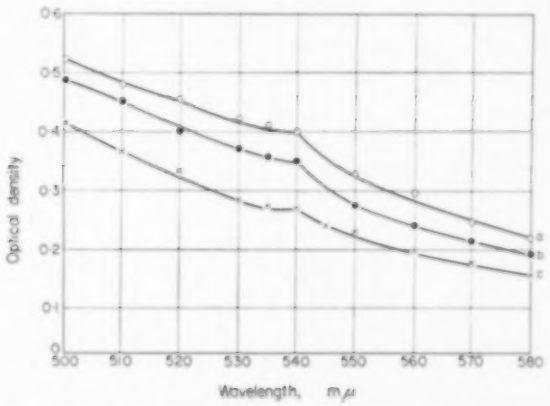

FIG. 1. Absorption spectrum of: extracts of (a) house fly eggs, (c) milkweed bug eggs, and (b) reagent ACh reacted with hydroxylamine–ferric chloride.

the absorption spectrum obtained in the egg extracts was identical with reagent ACh and shows the characteristic shoulder at 540 mμ (CHEFURKA and SMALLMAN, 1956), thus confirming that the pharmacologically active substance gives the chemical test for ACh.

As a final means of identification of ACh, extracts of eggs at the above stages of development were subjected to paper chromatography. Only one choline ester

was detectable in the extracts. Table 3 shows that this ester has a R_f comparable to that of reagent ACh subjected to the same treatment as the extract.

When the areas of chromatograms corresponding to the identified R_f of ACh were eluted and bioassayed with frog's rectus abdominis muscle, they showed the

TABLE 3—PAPER CHROMATOGRAPHY OF ACH

Solvent	Extract		
	ACh	House fly R_f	Milkweed bug R_f
Butanol–acetic acid–water; 4 : 1 : 5	0·65	0·66	0·65
Butanol–saturated with water	0·46	0·46	0·45
Butanol–ethanol–acetic acid–water; 8 : 2 : 1 : 3	0·64	0·64	0·63

expected pharmacological activity. Furthermore, the fact that the unknown was hydrolysed either by purified bovine ChE or by alkali provided additional evidence for the identification of ACh. The results taken *in toto* would appear to be firm identification of ACh in the eggs of the house fly and milkweed bug.

There is approximately twice as much ACh in the eggs of the house fly as in the milkweed bug at the later stages of development. The apparent cause of such a difference in the amounts of ACh in the eggs of house fly and milkweed bug cannot be determined by the present study, although it is worthwhile to note that a similar difference in amounts of ACh-like substance in the eggs of different species of Lepidoptera has also been recorded by YUSHIMA (1956), who observed ACh values of 0·5 mg/g in eggs of *Polia illoba*, 1·1 mg/g in the eggs of *Sedemia depravara*, and 1·0 mg/g in eggs of *Euxoa segitis*.

Cholinesterase

The comparative activity of ChE in the homogenates of the eggs of the house fly and the milkweed bug, at different stages of embryonic development, is shown in Table 4. These values, when compared with sources such as the house fly and bee head (WOLFE and SMALLMAN, 1956) and roach ventral nerve cord (CASIDA, 1955; COLHOUN, 1959a), are about 100 times lower. However, the contrast is not so great when the comparison is made with the entire bodies of insects (METCALF *et al.*, 1955), and are similar to the values reported for the house fly eggs (CASIDA, 1955) and for the milkweed bug egg (SMITH and WAGENKNECHT, 1956).

As with ACh, no ChE activity was detected either in eggs of the house fly or milkweed bug during the early stages of development. ChE activity first appeared in the egg of the house fly at 7 hr and then increased up to 11 hr, which is just prior to hatching. Similarly ChE activity in milkweed bug eggs was first observed on the fourth day and increased until the time of hatching. These findings on the occurrence of ChE activity only during the late stages of development confirm the findings of SMITH and WAGENKNECHT (1956) for ChE in milkweed bug eggs and

also agree closely with the results of CHINO and YUSHIMA (1954) for the eggs of *Chilo simplex*, *Barathra brassicae*, and *Bombyx mori*. Similar late appearance of ChE has also been noted in the eggs of *Diataraxia oleracea* (LORD and POTTER, 1954) and *Pieris brassicae* (POTTER *et al.*, 1957; DAVID, 1959).

TABLE 4—THE COMPARATIVE CHOLINESTERASE ACTIVITY IN EGG HOMOGENATES OF THE HOUSE FLY AND MILKWEED BUG AT DIFFERENT STAGES OF DEVELOPMENT

Species	Age	b_{20}
House fly	1–3–5 hr	Nil
	7 hr	6·9 ± 0·5*
	9 hr	10·0 ± 1·2
	11 hr	15·9 ± 1·3
Milkweed bug	1–2–3 day	Nil
	4 day	15·5 ± 1·5
	5 day	47·0 ± 4·7

* Standard deviation of the mean b_{20} = µl of CO_2 evolved by 125 mg of eggs per 30 min.

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Substrate specificity

Different substrates were used to determine the type of esterase which might be present in the eggs of the house fly and milkweed bug. These experiments were carried out using 11-hr-old eggs of the house fly and 5-day-old eggs of the milkweed

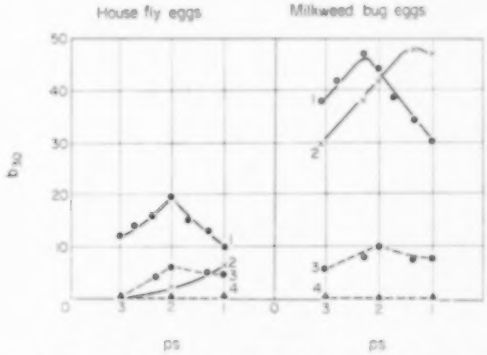

FIG. 2. Activity–pS curves for enzymatic hydrolysis by the house fly and milkweed bug egg ChE. b_{20} = µl of CO_2 evolved by 125 mg of eggs in 30 min. pS = −log molar concentration. 1—ACh; 2—AMeCh; 3—BuCh; 4—BzCh.

bug when the esterase activity was expected to be maximum. The resulting pS curves for different substrates after correcting for non-specific hydrolysis are shown in Fig. 2.

Regarding substrate specificity, it is seen that ChE from eggs of both sources reacted with ACh and BuCh to give characteristic bell-shaped curves. The activity of the enzyme towards BuCh was nearly one-quarter of that of ACh, and BzCh

was not hydrolysed by enzyme from both sources. These results show that the enzyme hydrolysing ACh found in both the species during the later stages of development can be termed 'true' or 'acetylcholinesterase'. However, a difference between the two species was observed when the activity of the enzyme for hydrolysis of AMeCh was determined. AMeCh was not hydrolysed to any extent by house fly eggs, whereas a measurable amount of activity was observed with milkweed bug eggs. Differences in the hydrolysis of AMeCh by ChE obtained from various insect eggs and adults has been noted (SMITH and WAGENKNECHT, 1956; Metcalf *et al.*, 1955).

The pS activity curve for milkweed bug eggs confirms the results of SMITH and WAGENKNECHT (1956) who found a similar pS optimum for the eggs of same species, and they also similar to those for milkweed bug heads (METCALF *et al.*, 1955). The occurrence of ChE in the house fly eggs confirms the results of CASIDA (1955), though in contradiction to his observations there was no evidence of the presence of two different enzymes hydrolysing ACh.

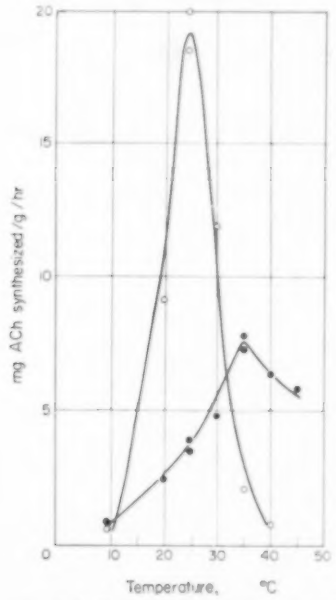

FIG. 3. Temperature optimum of choline acetylase from mouse brain (solid circles) and eggs of the milkweed bug (open circles).

Choline acetylase

Preliminary studies with extracts of eggs of the house fly and milkweed bug showed that more ACh was synthesized at 25°C than at 37°C, which supports the suggestion of HEBB (1957) that ChA obtained from invertebrates may have a lower temperature optimum than that obtained from vertebrates. The results in Fig. 3 indicate the rate of synthesis of ACh by ChA in the extracts of milkweed bug

and mice brain at different temperatures. A distinct difference in the temperature optimum for ChA from two sources is evident. The temperature optimum for house fly eggs is similar to that of the milkweed bug eggs. COLHOUN (1958) has found the temperature optimum for ChA extracted from house fly heads and the nerve cord of *Periplaneta* to be 30°C. The optimum for eggs of the house fly and milkweed bug is slightly lower. Therefore, all experiments on the synthesis of ACh by ChA of eggs were carried out at 25°C.

TABLE 5 (a)—*In vitro* CHOLINE ACETYLASE ACTIVITY (EXPRESSED AS mg/g/hr AT 25°C) OF EGGS OF THE MILKWEED BUG AT VARIOUS STAGES OF DEVELOPMENT (mg OF ACh SYNTHESIZED/g EGGS/hr)

Experiment No.	Age (days)				
	1	2	3	4	5
1a	0	5·2	10·0	20·0	17·0
2a	0	2·0	4·8	17·6	12·0
3b	0	6·0	11·0	22·0	18·0
4b	0	5·5	10·5	21·0	16·5

a—acetylcholine measured by chemical method.
b—acetylcholine measured by frog biological assay.

TABLE 5 (b)—*In vitro* CHOLINE ACETYLASE ACTIVITY (EXPRESSED AS mg/g/hr AT 25°C) OF HOUSE FLY EGGS AT VARIOUS STAGES OF DEVELOPMENT (mg OF ACh SYNTHESIZED/g EGGS/hr)

Experiment No.	Age (hr)				
	3	5	7	9	11
1a	0	1·6	10·0	16·0	12·0
2b	0	2·0	11·0	17·6	12·5
3b	0	2·2	10·8	18·0	12·0

a—ACh formed was measured by chemical method.
b—ACh formed was measured by frog rectus abdominis muscle.

Occurrence of choline acetylase during embryogenesis of the house fly and the milkweed bug

The results obtained regarding the appearance of ChA in eggs of the house fly and the milkweed bug are shown in Table 5. It is clear that the ChA is absent during the early stages of development both in the house fly as well as in the milkweed bug but precedes the appearance of ACh and ChE. In eggs of the house fly the ChA activity was first observed at 5 hr and the amount of enzyme increased steadily until just before hatching, when a slight decrease in activity was observed. Similarly, in eggs of the milkweed bug ChA activity was first detected on the second day of embryonic development and increased in amount until just before

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hatching and then declined slightly, as found with eggs of the house fly. Maximum ChA activity was observed in 9-hr-old eggs of house fly and 4-day-old eggs of the milkweed bug. The rate of synthesis of ACh by ChA at 9 hr of development was determined as 16 mg/g/eggs/hr. A similar high rate of synthesis of ACh was observed in the 4-day-old eggs of the milkweed bug.

These findings on the occurrence of ChA in developing insect eggs agree essentially with those of YUSHIMA (1957), who determined the appearance of ChA activity in the developing eggs of *Chilo simplex* and *Mamestra brassicae*, and that of CHINO (1957) on *Bombyx mori* eggs.

However, the values reported by these workers were very low; the highest values obtained for the synthesis of ACh at the late stages of development, when the activity of ChA was observed to be maximum, were as follows: 66 μg ACh/g eggs/hr in *Chilo simplex*, 500 μg/g/hr in *Mamestra brassicae*, and 360 μg/g/hr in *Bombyx mori*. An evaluation of the technique of these authors shows that the low rate of synthesis of ACh by ChA from these eggs when compared to the high rate found in the present study may be explained by the absence of certain cofactors in the synthesizing medium. The medium did not contain CoA, which as shown by SMALLMAN (1956) is rate limiting in ACh synthesis. Furthermore, apparently no precautions were taken to avoid the acetate activating enzyme from becoming rate limiting. Finally, all experiments were carried out at 37·5°C, which may partially inactivate the ChA of the species of eggs under investigation.

DISCUSSION

Establishing proof of the cholinergic system would require the demonstration of the substrate ACh and of the synthesis and hydrolysis enzymes ChA and ChE. Reports of pharmacologically active substances detected in the eggs of a number of lepidopterous insects by the use of frog's rectus abdominis muscle have been published by CHINO and YUSHIMA (1953), YUSHIMA and CHINO (1953), and YUSHIMA (1956a, b). However, as pointed out by CHANG and GADDUM (1933) such evidence by itself is inadequate and indicates only an ACh-like substance. In the present study the pharmacologically active substance present in the eggs of the house fly and milkweed bug, which activated eserinized frog's rectus abdominis muscle preparation, was identified by its chemical reaction with hydroxylamine–ferric chloride (HESTRIN, 1949) and by paper chromatography, and it was shown to be identical with ACh. Of importance is the evidence that only one choline ester was detectable by the above techniques, and in this manner the results are comparable to those found for adult insects by AUGUSTINSSON and GRAHN (1954), CHANG and KEARNS (1955), and CHEFURKA and SMALLMAN (1956).

Following the demonstration of ACh, evidence was obtained for the occurrence of both ChE and ChA. No attempt was made to determine the occurrence of aliphatic or aromatic esterases, although aromatic esterase is known to occur in the eggs of *Diataraxia oleracea* and *Ephestia kühniella* (LORD and POTTER, 1954). By the use of different substrates, some information as to the substrate specificity of the hydrolysis enzyme was obtained, which indicated that the aliphatic esterase

was absent and the aromatic esterase, if present, did not contribute significantly towards the hydrolysis of ACh. As a result, the hydrolysis enzyme was termed 'true' or acetylcholinesterase. A totally fortified homogenate was used for the demonstration of ChA activity. This method was much superior to that used by SMALLMAN (1956) and FRONTALI (1957) for tissues of adult insects. It was found that under these conditions of assay the temperature optimum for *in vitro* synthesis of ACh by ChA of insect eggs was approximately 25°C. These results may provide an explanation for the low rate of synthesis of ACh observed in other insect eggs (CHINO, 1957; YUSHIMA, 1957). Our data taken *in toto* lead to the conclusion that the eggs of the house fly and milkweed bug contain all three components of the cholinergic system.

During development, it was observed that ACh, ChE, and ChA appeared in the eggs of both species in an identical sequence, although the house fly embryo completed its development in 11 hr and the embryo of the milkweed bug required 5 days. In eggs of the house fly, ChA occurs first, then ChE, and finally ACh. Eggs of the milkweed bug differed in that ChE and ACh were found to appear simultaneously. There are also differences between eggs of the house fly and milkweed bug when the amounts of ACh, ChE, and ChA are compared. The house fly eggs have a higher ACh content, but eggs of the milkweed bug had more ChE and ChA when compared upon a basis of egg weight.

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It is difficult to ascertain from the present work where these substances are located in the embryo. However, if for comparison reference is made to the work of FORMIGONI (1954) on the embryology of house fly and BUTT (1949) on the embryology of milkweed bug, it becomes apparent that the appearance of ChA coincides with the appearance of neuroblasts. Therefore it is possible that the developing neuroblasts are the source of ChA.

The main objective in this study has been to establish as accurately as possible whether all components of the cholinergic system occur in insect eggs; this seemed pertinent to the hypothesis that ACh is a synaptic mediator in adult insects and to the corollary assumption that the organophosphate insecticides kill insects by inhibiting ChE so that ACh normally destroyed by the enzyme accumulates in the nervous tissue. The demonstration of ACh and the enzymes required for its metabolism in insect eggs, together with the well-known evidence of existence of these components in the nervous tissues of adult insects, indicates that ACh may be the most likely candidate for the role of synaptic mediator in insects as in vertebrates.

SUMMARY

(1) The occurrence of ACh, ChE, and ChA was demonstrated in eggs of the house fly and milkweed bug.

(2) The identity of ACh was established by means of bioassay, paper chromatography, and its reaction with hydroxylamine–ferric chloride. The hydrolysis enzyme was found to be 'true' or acetylcholinesterase.

(3) During development ChA was detected at 5 hr in eggs of the house fly and at 2 days in milkweed bug eggs. ChE and ACh were detected at 7 and 9 hr respectively in eggs of the house fly, but appeared simultaneously at 4 days in eggs of the milkweed bug.

(4) Concomitant with embryogenesis the amounts of ACh, ChE, and ChA increased progressively until hatching. In eggs of both species the levels of ChA decreased slightly prior to hatching.

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A BRIEF SURVEY OF THE EFFECTS OF POTENTIAL ANTIMETABOLITES AND ENZYMES ON THE DEVELOPMENT OF GIANT SILKMOTHS

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Abstract—More than 150 common purines, pyrimidines, purine and pyrimidine derivatives, and vitamin analogues were analysed for effects on insect growth and development. Compounds were injected into diapausing pupae of *Samia cynthia* and into pupae of *Callosamia promethea* just prior to the initiation of adult development. Most compounds were ineffective (e.g. uracil, 8-azaxanthine) or toxic (e.g. 6-methyl uracil, hypoxanthine). However, two classes of compounds had striking morphogenetic effects: the methyl xanthines (caffeine, theophylline, and theobromine) and barbituric acid and some of its analogues.

The methyl xanthines prevented initiation of adult development but were not toxic to pupae, many of which lived more than 2 months after injection. These compounds specifically attacked the insect's central nervous system, causing extensive degenerative changes in the brain and segmental ganglia. They prevented adult development by damaging neurosecretory mechanisms within the brain.

Barbituric acid and certain of its analogues (e.g. 5-amino uracil, 4,6-dihydroxypyrimidine, 1,3-dimethyl uracil, 6-methyl thiouracil) caused striking changes in the epidermis of developing adults but seemed to have little effect on the rest of the insect. The adults that emerged were almost devoid of scales, and the scales that did form were small and misshapen.

Several proteases, esterases, and nucleases were also assayed for effects on development. Ribonuclease proved especially interesting, because in sublethal doses it caused abnormalities of the epidermis strikingly similar to those produced by barbituric acid. These results, and other evidence, suggest that both barbituric acid and ribonuclease interfere with the RNA metabolism of epidermal cells.

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INTRODUCTION

THE life cycle of many insects is interrupted by a diapause during which development ceases and respiration falls to very low levels (SCHNEIDERMAN and WILLIAMS, 1953; LEES, 1955). In giant silkmoths of the family Saturniidae, this diapause occurs promptly after pupation and usually lasts for at least 5 months at room temperature. Few cell divisions, if any, occur in most tissues of the diapausing pupa (SMITH and SCHNEIDERMAN, 1954). When diapause ends, adult development begins and the adult moth is fabricated from the pupa. The mechanism controlling these growth processes is endocrinological and centres in the brain. Neurosecretory cells within the brain release a tropic factor, the brain hormone, which stimulates the prothoracic glands to secrete a growth and moulting hormone, the prothoracic gland hormone. In the presence of the

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prothoracic gland hormone, the cells of the insect grow and differentiate; in its absence they remain quiescent. When neurosecretion ceases, the prothoracic glands cease secreting, growth soon stops, and diapause supervenes.

Thanks to the skilful experiments of WILLIAMS (1946, 1952), this transition from no growth to active growth can be controlled at the convenience of the investigator by simple surgical manœuvres or by temperature treatment. The experimenter can turn the growth processes on and off at will, and for this reason the pupal silkworm and other diapausing insects provide excellent objects for examining biochemical differences between growing and non-growing cells. Indeed, several studies have already been performed on the enzymatic differences between diapausing and developing insects including silkworms (cf. for example BODINE and BOELL, 1934; WILLIAMS, 1951; SHAPPIRIO and WILLIAMS, 1957; WIGGLESWORTH, 1957; KURLAND and SCHNEIDERMAN, 1959; and review by LEES, 1956). Yet, except for nutritional studies (cf. extensive reviews by LEVINSON, 1955; FRIEND, 1958; HOUSE, 1958) only limited examination has been made of the effects of growth factors on the development of insects (FRAENKEL, 1943; FRAENKEL and PRINTY, 1954; WILLIAMS, 1953; also review by ASCHER and LEVINSON, 1953; and summary by SPECTOR, 1957). Even fewer studies have made use of growth factor analogues (VILLEE and BISSEL, 1947; VILLEE and LOWENS, 1948; SCHNEIDERMAN *et al.*, 1953; also summary of antivitamins in insect nutrition by LEVINSON and BERGMANN, 1959). This is perhaps surprising when one recalls the numerous studies on the effects of antimetabolites on vertebrate embryos which have provided a useful insight into the mechanisms of embryonic development and differentiation, particularly in such notable experiments as the blocking of lens induction (NIU, 1956) and the demonstration of the role of phenylalanine in neural crest differentiation (WILDE, 1956, 1959); see also BIEBER *et al.* (1952), BIEBER (1954), GUSTAFSON and HORSTADIUS (1955, 1956), HERMANN *et al.* (1955), WEISZ (1955), and reviews by BARTH (1957) and SHIVE and SKINNER (1958).

The present study was undertaken to assess the kinds of information antimetabolites might provide about insect growth and development. To this end, the effects of a selected group of potential antimetabolites was appraised on giant silkmoths during pupal diapause and at various stages of adult development. Several compounds were found which produced specific morphogenetic effects and have provided useful tools for analysing insect development. Brief reports of some of these findings have already appeared (BLAUSTEIN and SCHNEIDERMAN, 1958a, b, 1959). This preliminary survey will consider principally purine, pyrimidine, and vitamin analogues and enzymes. The action of steroids, amino acids, antibiotics, and carcinogens will be reported upon separately.

MATERIALS AND METHODS

1. *Experimental animals*

Experiments were performed on pupae of two closely related Saturniid moths, *Callosamia promethea* (1·5–2·5 g) and *Samia cynthia* (1·5–3·5 g). The pupae were in two physiological states: (a) 'Unchilled diapausing Cynthia pupae' which were

obtained from collectors in early fall and stored at 25°C. In our experience, this species never spontaneously initiates adult development without a period of chilling, and at 25°C will remain in permanent diapause for 18 months or more when they eventually die. (b) 'Previously-chilled Promethea pupae' which were collected in nature and stored at 5–8°C for 5–9 months prior to use. This prolonged exposure to low temperature brings about physiological changes within the pupal brain which enable certain neurosecretory cells to secrete brain hormone. This results in the initiation of adult development about 12–18 days after the pupae are placed at 25°C. In most experiments the chilled Promethea pupae were injected on the same day they were removed from the cold. In a few experiments, in addition, some chilled pupae were placed at 25°C for about 2 weeks and then injected with the same compounds. Since the two groups behaved identically, the results were combined.

Most compounds were injected into both diapausing Cynthia pupae and into previously-chilled Promethea pupae about to initiate adult development. In addition a sufficient number of experiments were performed on diapausing Promethea pupae to show that they behaved identically to diapausing Cynthia and could be used interchangeably in experiments of this kind. Hence, when differences in response appeared between diapausing Cynthia pupae and previously-chilled Promethea pupae, they were safely attributable to differences in the physiological states of the pupae and not to species differences.

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2. *Experimental procedures*

About 150 compounds were tested for biological activity. Commercial preparations were used in all cases, except for isobarbituric acid, which was provided by Dr. GEORGE S. KIDDER. Most compounds were dissolved in 0·1 M phosphate buffer at pH 7·0. When the pH of the resulting solution deviated from 7·0, potassium hydroxide of hydrochloric acid was added until neutrality was reached. This procedure may have been unnecessary, for in control tests injections of 0·1 ml of acids as strong as pH 4 had no apparent effect on adult development. Solutions ranged in concentration from 1 to 100 mg/ml. A few substances that failed to dissolve (e.g. benzimidazol, indole) were simply suspended in the buffer, and in these cases the amounts injected were not precise.

Pupae were anaesthetized with carbon dioxide and injected in the thoracic dorsum to one side of the midline. The amount injected averaged 0·03–0·05 ml/g live wt. Immediately following injection, the wound was sealed with melted wax and the animal stored at 25°C and 60–70 per cent humidity. Uninjected pupae and pupae injected with phosphate buffer served as controls for each series of experiments. The animals were observed at 3–4 day intervals and the progress of development recorded. A modification of the time-table for development devised by SCHNEIDERMAN and WILLIAMS (1954) guided observations during the 18-day period of adult development. Notice was taken of the following effects: (1) toxicity; (2) delaying or prevention of the initiation of adult development; (3) gross morphological abnormalities in adult moths (including deformities of the antennae, eyes,

TABLE 1—NON-TOXIC COMPOUNDS

Compound	Maximum dose injected (mg/g live wt.)	Compound	Maximum dose injected (mg/g live wt.)
Adenine	5·9	Glucoasorbic acid*	2·0
Adenine sulphate	6·4	Glutathione (reduced)	6·7
Adenosine-5′-phosphate*	2·0	Guanine hydrochloride	6·3
Adenosine diphosphate*	2·0	Guanine sulphate	7·7
Adenosine triphosphate*	2·0	Guanylic acid	7·7
Allyl glycine	7·7	Indole*	2·0
Aminoacetic acid	7·1	Inosine*	6·6
4-Amino-2, 6-dihydroxy pyrimidine	6·3	Isoguanine sulphate	8·3
2-Amino-4, 6-dioxy pyridimine	6·7	d-Leucine*	2·0
Aminotyrosine dihydrochloride*	1·0	dl-Leucine*	2·0
6-Amino uracil	6·3	Mannose-6-phosphate*	3·9
l-Asparagine	9·1	6-Mercaptopurine hydrate	7·7
8-Aza-2, 6-diaminopurine sulphate	10·0	Methionine sulphoxide*	2·0
8-Aza xanthine	8·3	2-Methyl-4-amino-5-aminoethyl pyrimidine hydrochloride	8·3
γ-Benzene hexachloride	2·0	5-Methyl orotic acid	6·2
5-Bromouracil	7·1	5-Nitrobarbituric acid	7·3
Butabarbital (sodium)*	0·83	5-Nitro uracil	7·7
Canavanine sulphate*	2·0	Oxythiamine*	2·0
Cytidine*	2·0	Pantoyl taurine (sodium)*	6·7
Cytidine sulphate*	2·0	Phenobarbital*	7·7
Cytosine	5·9	Protamine nucleinate*	7·7
Desoxyribonucleic acid (sperm)*	5·3	Protamine sulphate	7·1
dl-Desthiobiotin*	2·0	Pyrimidine-3-sulphonic acid	9·0
Diallyl barbituric acid*	0·75	Ribonucleic acid*	5·3
2, 4-Diamino-6-hydroxy pyrimidine sulphate	8·3	*d* (-) Ribose	6·6
4, 6-Diamino-2-thio pyrimidine*	5·0	Ribose-5-phosphate*	3·6
4, 5-Diamino uracil sulphate	7·7	Sodium desoxyribonucleate*	7·2
Dihydrocholesterol*	2·0	Sulfanilamide	7·7
Dihydrothymine	7·7	Thiamine hydrochloride*	7·0
2, 4-Dioxy-6-amino pyrimidine	6·4	β-2-Thienylalanine*	2·0
Diphosphopyridine nucleotide	7·1	2-Thio-6-oxypurine	6·2
2, 8-Dithio-6-oxypurine	6·4	2-Thiouracil-5-carboxylic acid	6·6
Estradiol*	2·0	Thymidine*	5·0
dl-Ethionine*	2·0	Thymine	7·7
Fructose-1, 6-diophosphate*	6·7	α-Tocopherol quinone*	7·1
Galactose-6-phosphate*	2·0	Uracil	5·0
		Uracil-4-acetic acid	7·7
		Uracil-5-carboxylic acid	7·1
		Uric acid*	3·9
		Uridine	7·7
		Xanthine*	6·7
		Xanthine (monosodium)	7·3

At least twelve chilled pupae and four diapausing pupae injected with each compound except for compounds marked with an asterisk.

* Four chilled pupae but no diapausing pupae were injected.

TABLE 2—TOXIC COMPOUNDS

Compound	Approximate MLD† (mg/g live wt.)	Approximate lethality time at MLD (days)
3-Acetyl pyridine	2·1	40
Adenosine	1·3	40
2-Amino-4-methyl pyrimidine	0·36	42
2-Amino pyrimidine	1·7	40
2-Amino-4, 6-pyrimidinedione	6·7	31
Amobarbital*	2·0	5
8-Aza-guanine‡	6·3	31
2-Chloro-4-aminobenzoic acid*	2·0	50
8-Chloroxanthine	5·6	10
Cytidylic acid	0·35	30
Desoxypyridoxine hydrochloride	2·0	3
2, 6-Diamino purine sulphate	1·4	33
Diazouracil	1·5	28
2, 4-Dichloropyrimidine	1·6	29
Dicoumarol*	2·0	2
2, 4-Dithiopyrimidine	0·32	20
Fructose-6-phosphate	1·4	42
Glucose-6-phosphate	0·32	19
Guanine	1·3	64
Guanosine	0·38	37
Hypoxanthine	1·3	37
Isobarbituric acid	7·7	16
Isocytosine	1·4	42
5-Methyl thiouracil	2·0	24
6-Methyl uracil	5·9	60
Orotic acid	7·1	29
Pentobarbital*	4·0	5
dl-Phenyllactic acid	5·6	36
Phosphoglyceric acid	0·33	42
α-Picolinic acid (hydrochloride)	0·25	25
Pyrithiamine (hydrobromide)	2·0	29
Seconal*	1·3	5
2, 4, 5, 6-Tetra amino pyrimidine sulphate	5·6	53
β-Thienyl serine	2·0	20
2-Thio-4, 6-dioxypyrimidine	5·0	21
Thiouracil	1·3	31
2, 4, 5-Triamino-6-hydroxypyrimidine sulphate	7·1	30
2, 4, 6-Triamino pyrimidine	6·3	58
4, 5, 6-Triamino pyrimidine sulphate	6·6	30
Uramil	5·6	22
Vinbarbital	1·5	4
Xanthosine	3·0	28

At least twelve chilled pupae and five diapausing pupae were injected with each compound except those marked with an asterisk.

* A minimum of four chilled pupae but no diapausing pupae were injected.

† Minimum lethal dose to kill 90 per cent of the animals. These values are, of course, only approximate, especially for cases where only a few insects were studied.

‡ About 30 per cent of pupae injected with sub-lethal doses of 8-aza-guanine (1·3–5·9 mg/g live wt.) failed to initiate adult development but survived for more than 60 days.

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external genitalia, scales, pigmentation patterns, and general body form); and (4) decreased activity or viability. When the preliminary result suggested further analysis, dissection and examination of internal organs, histological studies, and further experiments were conducted. These will be described in detail elsewhere. Over a 3 yr period a total of 2500 pupae were individually examined.

RESULTS

The compounds tested fell into three fairly distinct groups:

(1) Non-toxic compounds which had no visible effects on adult development or on diapausing pupae at doses up to 5 or 10 mg/g live wt. (Table 1).

(2) Toxic compounds which prevented adult development and caused death within 60 days of injection (Table 2).

(3) Compounds which had specific effects on adult development (Table 3). These compounds were of three kinds: (a) substances which caused abnormal scale formation in adults, (b) substances which prevented the initiation of adult development but did not cause death within 60 days after injection, and (c) substances which were far more toxic to diapausing pupae than to previously-chilled pupae.

The results summarized in Tables 1 and 2 require little amplification. It can easily be seen that most compounds tested were without effect and a lesser number were toxic. An exception was 8-aza-guanine (Table 2). About 30 per cent of animals injected with sub-lethal doses of this substance (1·3–5·9 mg/g live wt.) failed to initiate development but survived for more than 60 days after the injection.

All of the substances listed in Table 3A inhibited scale formation during adult development but seemed to have little effect on the rest of the insect. The most potent of these compounds appeared to be barbituric acid, and its effects were studied in detail. In animals treated with barbituric acid or these other compounds, scales were scanty and were short and club-shaped rather than long and flat. Moreover, they lacked the fine structure of normal scales: the crown-like distal edge, characteristic of normal scales, was absent, and the rib-like structure of the normal scale was replaced by an amorphous granular wall. The effect appeared to be a quantitative one. After doses of 0·46 mg/g live wt., only a few scales formed and all of these were stunted as described above. At lower doses (e.g. 0·30–0·40 mg/g live wt.) an intermediate condition prevailed where normal scales covered the tip of the abdomen, a mixture of normal and abnormal scales covered the remainder of the abdomen, and truly club-shaped scales, only one-third normal size, covered the wings and thorax.

In addition to the effects on scales themselves, the adult cuticle of treated animals was abnormally thin although positive chitosan tests revealed the presence of chitin. Dissection failed to reveal abnormalities in the viscera, muscles, or sense organs and perfectly normal looking eggs were produced by females. The only other discernible abnormality was a decrease in the enzymatic activity of the moulting fluid and an impairment of the process of moulting fluid resorption. Thus, moulting fluid failed to fully digest the old pupal endocuticle and was not reabsorbed

TABLE 3—COMPOUNDS WITH SPECIFIC EFFECTS ON ADULT DEVELOPMENT

A. *Compounds causing abnormal development of scales*

Compound	Approximate MED (mg/g live wt.)*
Barbituric acid	0·46†
4, 6-Dihydroxy pyrimidine	0·7
5-Amino uracil	4·0
1, 3-Dimethyl uracil	4·6
6-Methyl thiouracil	4·6
Ribonuclease	0·02–0·05

B. *Compounds preventing the initiation of adult development for at least* 60 *days*

Compound	MED (mg/g live wt.)	Per cent of pupae injected with MED that failed to initiate adult development
Caffeine	0·28	90
2, 5-Diamino 4, 6-pyrimidine-dione	4·4	71
2, 5-Diamino pyrimidine	1·3	83
Theobromine‡	1·2	30
Theophylline	0·35	100

C. *Compounds whose MLD differed by a factor of at least* 10 *in previously-chilled and in diapausing pupae*

Compound	MLD for previously-chilled pupae	MLD for diapausing pupae
Benzimidazol	3·6	0·30
Glucose-1-phosphate	5·3	0·30
Quercetin§	3·1	0·24
2-Thiocytosine	6·7	0·28
Adenosine-3-phosphate	6·3	0·35

At least twelve animals were tested with each compound and more than 100 were used in a few cases.

* Minimum effective dose. Doses below MED permitted normal development.

† This is MED for a partial effect. A dose of 0·8 mg/g live wt. is necessary for complete inhibition of scale formation.

‡ Theobromine was not as effective as the other compounds listed.

§ Quercetin prevented the initiation of adult development (but permitted animals to survive for at least 60 days) in about 20 per cent of chilled pupae injected with 2 mg/g live wt.

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at the end of adult development. As a result the adults were unable to eclose. Viability, however, was normal, and trapped within their leathery pupal cuticles these strange creatures survived as long as normal adults (about 10 days), releasing their meconium while enclosed. The other pyrimidine analogues listed in Table 3A had effects very similar to those of barbituric acid but required larger doses. A histological description of the effects of barbituric acid and its analogues on scale formation, and a biochemical analysis of its mode of action, will be presented in a later communication.

The second group of active compounds were methylxanthines and 2, 5-diamino pyrimidine (Table 3B) which prevented chilled pupae from initiating adult development and thus, in effect, enforced an artificial diapause. Animals injected

TABLE 4—EFFECTS OF ENZYMES

Enzyme	Approximate MLD to kill 90% (mg/g live wt.)	Approximate lethality time at MLD (days)
Lysozyme	Non-toxic up to 2·0 mg	
Lipoxidase	Non-toxic up to 8·3 mg	
Cytochrome *c*	2·0	4
Desoxyribonuclease	0·025	31
Ficin	0·04	14
Hyaluronidase	0·07	30
Lipase	0·15	15
Pan-protease	0·33	16
Papain	1·4	14
Ribonuclease	0·06	31

At least twelve chilled pupae and four diapausing pupae were injected with each enzyme.

with a minimum effective dose of any one of these compounds survived as pupae for at least 60 days (and often as long as 125 days) before dying. Dissection of these animals revealed all the organs to be quite normal except for nervous tissues. The protocerebrum appeared peculiarly transparent, and histological examination of the brain and ventral ganglia revealed degeneration of neuroglial elements and of fibre tracts as well as pycnosis of some brain cells. Muscular function was of course impaired because of damage to the nervous system, and the animals were flaccid. The heart beat in the intact pupa varied from an occasional single beat in a 10 min period to no beats at all over 45 min of observation.

The final group of compounds listed in Table 3C were far more toxic for diapausing than for chilled pupae. In every case the minimum lethal dose differed by a factor of at least 10. In addition, in occasional experiments, sub-lethal doses of quercetin prevented the initiation of adult development for at least 60 days without killing the animals.

The effect on development of injecting various enzymes is recorded in Table 4. A few had no effect even at very high doses, e.g. lipoxidase and lysozyme. Most, however, were very toxic, even in small amounts and for some, whose substrates

are almost exclusively intracellular (e.g. ribonuclease and desoxyribonuclease), the toxicity suggested cell penetration by these proteins. It is of special interest that sub-lethal doses of ribonuclease (0·2–0·06 mg/g live wt.) had effects remarkably similar to barbituric acid.

DISCUSSION

(1) *Inactive compounds*

Little need be said about most of the inactive compounds. It is not surprising that some of them like ATP, *d*-ribose, and uracil which are normal cell constituents were without effect. Some of the compounds like thienylalanine, ethionine, and oxythiamine are analogues of one or more normal metabolites (i.e. phenylalanine, methionine or thymine or nor-leucine, and thiamine) and are known to inhibit growth and other synthetic processes in vertebrates, higher plants, fungi, protozoa, and bacteria (MUNIER and COHEN, 1956; THIMANN and RADNOR, 1955; SUZUKI and KISHIDA, 1956; GROSS and TARVER, 1955). Possible explanations for the inactivity of such potential antimetabolites in insects (or in other organisms) are manifold and have been adequately summarized by WOOLLEY (1952, 1959), RHOADS (1956), and SHIVE and SKINNER (1958). Whether the apparent insensitivity of these insects to compounds like thienylalanine and 5-bromouracil, which are potent inhibitors of growth in other organisms (for example HAM and EAKIN, 1957), and to γ-benzene hexachloride which is an insecticide, stems from fundamental biochemical differences, from the presence of an excess of the normal metabolite, or from permeability differences, detoxification, etc., cannot be determined without a most careful study like that conducted by O'BRIEN and FISHER (1958) for various pharmacological reagents.

(2) *Toxic compounds*

The large number of toxic substances prevents a detailed analysis of their probable modes of action (cf. WOOLLEY, 1952, 1959). Some compounds like thienylserine, thiouracil, and hypoxanthine are most likely inhibiting reactions which engage one of their normal analogues, namely serine, uracil or thymine and xanthine. This is surely true also of most of the antivitamins (although reversal of the toxic effects by a natural metabolite is ordinarily required for final proof). Since analogues of nicotinic acid, pyridoxine, and thiamine are effective inhibitors of adult development, these vitamins doubtless play a role in the metabolism of giant silkworms. This result is consistent with nutritional studies which have revealed a dietary requirement in all insects studied for these three B vitamins as well as for riboflavine and pantothenic acid and probably also for folic acid and biotin (HOUSE, 1958; LEVINSON and BERGMANN, 1959).

In the case of nicotinic acid analogues our results revealed that 3-acetylpyridine and α-picolinic acid were considerably more effective than pyridine-3-sulphonic acid which was tolerated in large doses. Identical results have been reported by LEVINSON and BERGMANN (1959) in their recent studies of antivitamins in the nutrition of the house fly *Musca vicinia*. Moreover, these workers also showed that neopyrithiamine and desoxypyridoxine inhibited the development of their

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insect just as they inhibited the development of Promethea. Inhibition of larval growth by 3-acetylpyridine and desoxypyridoxine has also been shown in nutritional experiments with the commercial sikworm, *Bombyx mori* (SHYAMALA and BHAT, 1958). In contrast to the effectiveness of these B vitamin analogues, pantoyltaurine, which is a well-known inhibitor of pantothenic acid in many organisms including *M. vicinia* (LEVINSON and BERGMANN, 1959) and *Periplaneta americana* (SIEBURTH and MCLAREN, 1953), had no toxicity for chilled Promethea pupae even at relatively high doses.

The toxicity of normal purine and pyrimidine derivatives deserves some special mention. With one minor exception* it appears that the purine nucleosides—adenosine, guanosine, and xanthosine—were more toxic than the bases themselves or the nucleotides. This curious toxicity of purine nucleosides and other nucleic acid derivatives finds several parallels in other insects. Addition of RNA, DNA, adenine, guanine, or uracil to the diet inhibits the adult emergence of *Phormia regina* (BRUST and FRAENKEL, 1955) and *Pseudosarcophaga affinis* (HOUSE and BARLOW, 1957). The nucleic acids in these studies were probably toxic because they were hydrolysed in the gut to toxic end-products. The lack of toxicity of DNA and RNA in Promethea probably stems from the fact that these compounds are not readily hydrolysed when injected into the blood.

The toxic action, observed in the present study, of purine ribosides and of other compounds like glucose-6-phosphate and phosphoglyceric acid which are normal metabolites needs to be explained but finds many parallels in a variety of organisms; e.g. in rats phenylalanine and tyrosine can retard growth (BENTON *et al.*, 1956); in certain strains of *E. coli* valine is an antagonist (UMBARGER, 1957), in cultures of carrot tissues grown *in vitro* hydroxyproline is an antagonist of growth and synthesis (STEWARD and POLLARD, 1958). In insects, in addition to the reports cited above of inhibition by nucleic acid derivatives, there are numerous cases of inhibition by normal metabolites. Thus a variety of sugars including normal metabolites like galactose have been shown to inhibit the growth of *Tenebrio molitor* (FRAENKEL, 1955) and *P. affinis* (HOUSE, 1958), and glucose itself retards the growth of *Calliphora erythrocephala* (SEDEE, 1956).

The biochemical mechanisms whereby normal metabolites become toxic are manifold but among the most important are surely feedback inhibitions like those demonstrated by Magasanik and his co-workers for histidine biosynthesis, where histidine inhibits both the first enzyme in the pathway leading to histidine as well as the synthesis of histidine-forming enzymes (cf. review by MAGASANIK, 1957; MOYED, 1958). The feedback control of purine biosynthesis by product inhibition has been demonstrated in both bacteria (MAGER and MAGASANIK, 1958) and vertebrates (WYNGAARDEN *et al.*, 1958), and similar mechanisms doubtless occur in insects.

* In the entire series of more than ninety purines and pyrimidines the only result which seems inconsistent is the observation that in one experiment, involving eleven pupae, guanine was toxic at a final concentration of 1·3 mg, whereas in another experiment involving thirty-five pupae, guanine hydrochloride and guanine sulphate (which are for all purposes identical with guanine) were considerably less toxic. We have no explanation.

(3) *Substances which inhibit scale formation*

Only a few instances are known of chemicals which cause specific morphogenetic effects in insects and most of these involve the compound eyes. The best known effect is probably that of 'anti-Bar' substances which suppress the action of the Bar gene which inhibits the formation of facets in the eyes of *Drosophila*. Barbituric acid, uracil, uramil, acetamide, 1-methyl hydantoin, urea, histidine, adenosine, and xanthine are among the dozens of compounds, most with amide linkages, which are effective (KHOUVINE *et al.*, 1943; KAJI, 1954, 1955, 1956). Another well-known example is the effect on eye-colour in *Drosophila* of kynurenine and 3-hydroxykynurenine (BUTENANDT *et al.*, 1954). It is because such morphological effects seem to be rare that the action of barbituric acid and some of its analogues on scale-formation assumes special interest. These compounds inhibit scale-formation quite early in development, and the naked-looking adults which result are striking to behold. A cytological description of these effects will be reported subsequently, but for the present it is worth exploring the possible mode of action of barbituric acid.

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A first question concerns the biochemical role of barbituric acid. The fact that it differs from uracil solely by the presence of a hydroxyl group at the 6-position suggests that it may interfere in some way with uracil metabolism and hence with ribonucleic acid metabolism or with reactions which utilize uridine nucleotides such as polysaccharide synthesis* (HARTMAN and BUCHANAN, 1959). It will be shown elsewhere (BLAUSTEIN and SCHNEIDERMAN, 1961) that barbituric acid has no significant effect on cell division in the epidermis. This must mean that DNA synthesis is not a major site of action of barbituric acid and is consistent with the view that uracil and not one of the other pyrimidines is the target of this reagent. This opinion gains some support from the fact that other structural analogues of uracil (5-amino uracil, 1, 3-dimethyl uracil, 6-methyl thiouracil, and 4, 6-dihydroxy pyrimidine) inhibit scale-formation in the same way as barbituric acid. Moreover, the action of these compounds appears specific and a variety of related analogues of uracil have no effect (e.g. 5-hydroxy uracil (isobarbituric acid), 5-bromouracil, 5-nitrouracil, thymine, etc.). It is worth noting that barbituric acid is an intermediate in the degradation of thymine and uracil in certain organisms (HAYAISHI and KORNBERG, 1952) and in some systems it has been claimed to interfere with pyrimidine metabolism (e.g. *Drosophila* larvae (VILLEE and LOWENS, 1948); frog embryos (BIEBER, 1954)). For the present, the biochemical mode of action of barbituric acid in the insect remains uncertain.

A further problem is the apparent tissue-selectivity of barbituric acid. Why does it seem to attack only the epidermal cells? The answer may be that barbituric acid actually damages many cells in many tissues, but tissues other than epidermis can recover. This recovery probably takes place in large measure *not* by damaged cells recovering their function *but* through repopulation by undamaged cells. In the epidermis such repopulation is not possible since there seem to be

* Including possibly chitin synthesis if GLASER and BROWN's report (1957) of a uridine diphosphate requirement for chitin synthesis in certain fungi extends to insects.

virtually no reserve populations of epidermal cells in the pupa. When scale-stem cells in an area fail to secrete scales, that area is deficient in scales and never recovers. Such an explanation is consistent with our present understanding of the developmental capacities of various insect cells and is supported by observations on the effects of burns, wounds, and X-rays on the pupal–adult transformation.

Other agencies produce scale deficiencies which superficially resemble the effect of barbituric acid. The best known example occurs in the moth *Ephestia* where lack of linoleic acid in the diet causes moths to emerge with a deficiency of scales on the wings (FRAENKEL and BLEWETT, 1945, 1946). However, the missing scales are always found inside the cast exuvia. Hence, linoleic acid deficiency although it may produce adult moths deficient in scales does not act in any way like barbituric acid which inhibits the development of scales.

(4) *Substances which prevent development*

Caffeine (1, 3, 7-trimethylxanthine) and its relatives had a remarkable and specific action on the central nervous system of the pupa causing degeneration of the brain and segmental ganglia while sparing the other pupal tissues. Thus, apparently apart from its usual role as a pharmacological reagent, caffeine offers itself as a specific agent for selectively destroying the insect's nervous system. It is not without interest that structural analogues of caffeine like theophylline (1, 3-dimethylxanthine) and theobromine (3, 7-dimethylxanthine) exert similar effects on the insect. The order of decreasing effectiveness of these compounds, caffeine, theophylline, and theobromine, also parallels their decreasing pharmacological activity as stimulants of the vertebrate central nervous system and order of decreasing solubility in water (GOODMAN and GILMAN, 1955).

This degeneration of nervous elements without conspicuous action on the rest of the insect finds a parallel in the observations of WIGGLESWORTH (1941) on the effects of rather different kinds of substances, the pyrethrins. WIGGLESWORTH reports that when *Rhodnius* were exposed to pyrethrum, they were promptly paralysed, but often survived for up to 20 days. Histological examination disclosed extreme degeneration of ganglia, which appeared not unlike the degeneration encountered in the present study.

The mode of action of methylxanthines in the insect has not been determined. The fact that 2, 5-diamino pyrimidines and 8-azaguanine (Table 2) which are not very similar in structure to the methylxanthines have similar effects on the insect's nervous system makes the picture more perplexing. It may be that in the insect high doses of methylxanthines are acting as inhibitors of purine metabolism. Indeed, such a role for methylxanthines and 8-azaguanine as inhibitors of growth in *Tetrahymena* was demonstrated more than a decade ago by KIDDER *et al.* (1949). In addition there are numerous reports of methylxanthines interfering with a variety of synthetic processes in many kinds of cells including sea urchin gametes (CHENEY, 1956), plant cells (KIHLMAN and LEVAN, 1949), and micro-organisms (DEMEREC, 1949). Although the mechanism of action of the methylxanthines is not known, in insects, at least, they provide a novel way of destroying the central

nervous system, without causing obvious damage to other tissues. The application of this chemical deganglionation to studies of the trophic function of insect nerves (KOPÈC, 1923; NUESCH, 1952) might prove rewarding.

(5) *Compounds which were more toxic to diapausing pupae than to previously-chilled pupae*

The simplest explanation of these results is a quantitative difference between the intermediary metabolism of diapausing and chilled pupae. Earlier studies revealed that diapausing pupae are considerably more sensitive to 2, 4-dinitrophenol than developing adults, presumably because the diapausing pupae have only limited respiratory capacity (KURLAND and SCHNEIDERMAN, 1959). From the present experiments it seems likely that the greater toxicity of the several compounds for diapausing pupae stems from the fact that the target enzyme systems are present in only limited concentrations in the diapausing pupae compared to the previously-chilled pupae. The curious toxicity for diapausing pupae of glucose-1-phosphate—a normal cell constituent—remains obscure. The toxicity of normal metabolites when they are in the 'wrong compartment' needs to be examined further.

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(6) *Toxicity of enzymes*

The toxicity of most enzymes is perplexing. Some, such as the proteolytic enzymes, are capable of hydrolysing blood proteins and might in this way cause damage. Others like cytochrome *c* which one might expect to be innocuous proved strangely toxic. The two nucleases were also toxic, and since their substrates are intracellular it seems likely that they are able to penetrate cells and there exert their damage. This is not too surprising for a small protein like ribonuclease which has already been shown to enter many types of living cells of animals and plants and so to disturb cell function (CHANTRENNE, 1958). It is of special interest that in many (but not all) pupae, ribonuclease caused developmental anomalies similar to those produced by barbituric acid. This result is consistent with the suggestion offered above that barbituric acid may act by interfering with nucleic acid metabolism.

In summary, this preliminary survey appears to have uncovered eleven compounds which have specific morphogenetic effects on insects—nearly 8 per cent of compounds examined. Besides these compounds, nearly fifty other metabolites and potential antimetabolites were found to be toxic. This group includes analogues of three B vitamins, a variety of nucleic acid derivatives, and several normal metabolites. These results suggest that a more detailed survey might prove rewarding.

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THE NUTRITIONAL REQUIREMENTS OF LOCUSTS—II UTILIZATION OF STEROLS

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Abstract—The locusts *Schistocerca gregaria* (Forsk.) and *Locusta migratoria* L. are able to utilize cholesterol, cholesteryl acetate, β-sitosterol, and dihydrocholesterol (cholestan-3β-ol), but not stigmasteryl acetate, ergosterol, 7-dehydrocholesterol, cholestenone, or 7-oxocholesteryl acetate. From this it is concluded that as with other insects an OH group, which may be esterified, is needed at position 3 of the sterol nucleus for utilization to occur, whereas a ketone grouping at position 7 prevents utilization. Locusts differ from most other insects studied in that utilization appears to be prevented by a double bond at either position 7 or 22.

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INTRODUCTION

A DIETARY sterol is required by all insects hitherto examined, and in all cases cholesterol can fulfil this need. The ability to utilize other sterols varies considerably (NOLAND, 1954 a; LEVINSON, 1955), and it was early suggested that two types of utilization characteristic of zoophagous and phytophagous species could be recognized (FRAENKEL and BLEWETT, 1943). The phytophagous type was characterized on the basis of information derived from grain- and yeast-feeding insects, for appropriate studies were lacking for insects which eat the green parts of plants. Locusts are such insects, and this paper describes experiments on their ability to utilize various steroids.

MATERIALS AND METHODS

The two species of locust used, *Locusta migratoria* L. and *Schistocerca gregaria* (Forsk.), were kept in culture at the laboratories of the Anti-Locust Research Centre, London, and egg-pods from gregarious stocks were kindly supplied by this laboratory ready for hatching and were used in experiments the day they hatched. Experimental procedures and the preparation of synthetic diets have been described in the previous paper in the series (DADD, 1960). The composition of the basic diet used in the present group of experiments is given in Table 1.

RESULTS

The effectiveness or otherwise of steroids was determined by recording the growth and survival of hatchlings on diets in which cholesterol was replaced by the test substance. Mean growth curves for both species on diets containing

various concentrations of cholesterol are shown in Fig. 1. Little growth occurred with either species for concentrations up to 0·28 mg per gram of diet, whereas optimal growth was obtained over the range 0·84–5·6 mg per gram. By using test substances at concentrations of 1·4 mg per gram of diet, it could be assumed that growth to the adult stage would indicate extensive utilization, and that

TABLE 1—COMPOSITION OF THE BASIC DIET

Cellulose powder (Light and Co.)	15·0 g
Linoleic acid (B.D.H. technical grade)	0·2 ml
Cholesterol	50 mg
Salt mixture (Glaxo, DL–6)*	1·5 g
Sucrose	5·0 g
White dextrin (Hopkins and Williams)	5·0 g
Casein, fat and vitamin free (B.D.H.)	6·0 g
Bacteriological peptone (B.D.H.)	2·0 g
Egg albumen powder (B.D.H.)	2·0 g
Ascorbic acid	100 mg
Water, containing B vitamins in solution	10·0 ml
Vitamins:	
thiamine	25 μg/g diet
riboflavin	25 μg/g diet
nicotinic acid	100 μg/g diet
pyridoxine	25 μg/g diet
folic acid	25 μg/g diet
inositol	250 μg/g diet
Ca. pantothenate	50 μg/g diet
p-aminobenzoic acid	25 μg/g diet
biotin	1 μg/g diet
choline chloride	1250 μg/g diet

* For composition of this salt mixture see DADD, 1960.

unless active impurities amounted to more than 50 per cent, little growth would occur with a non-utilizable substance. Commercially obtained test steroids were of 95 per cent purity, and thus were unlikely to give rise to misleading results. Cholesterol, cholesteryl acetate, and ergosterol were supplied by B.D.H. Ltd.; all other steroids were obtained from Light and Co.

Growth data from experiments with both *Schistocerca* and *Locusta* hoppers are given in Tables 2 and 3. Various natural sterols and other steroid substances were first tested (Exp. 1). Of these, only cholesterol and β-sitosterol were utilized. Surprisingly, both ergosterol and stigmasteryl acetate were ineffective, although these had been utilized to some extent by all insects previously examined with the exception of the zoophagous species *Attagenus* and *Dermestes*. Sitosterol gave somewhat better growth than cholesterol with both species of locust, and this point was checked in the second experiment in which several derivatives of cholesterol were tested.

Of these, only dihydrocholesterol (cholestan-3β-ol) allowed good growth of the order obtained with cholesterol. Sitosterol was again slightly superior to

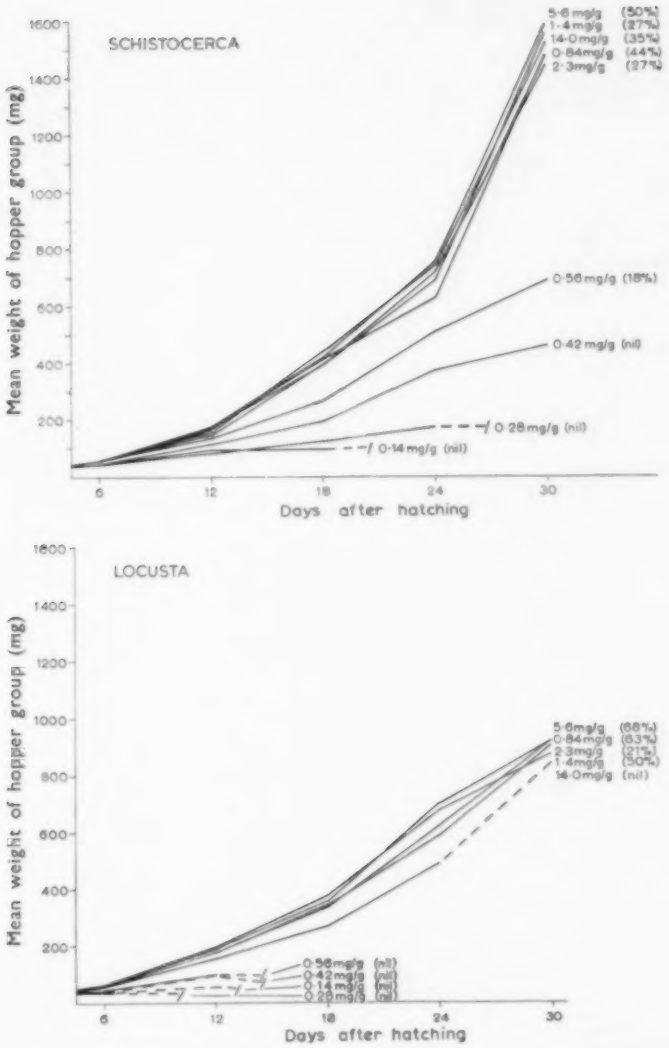

FIG. 1. Mean growth curves of groups of locust hoppers for various concentrations of cholesterol. Figures at the end of each curve show the concentration of cholesterol and (in brackets) the percentage of larvae alive at the sixth day to become adult. A broken line is used to indicate that 10 per cent or less of the larvae alive on the sixth day were surviving.

cholesterol with both species. As the stigmasterol and 7-oxocholesterol used in these experiments were supplied as acetates, some indication of the effect of esterification on utilization was sought by comparing growth on cholesteryl

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TABLE 2—GROWTH OF GROUPS OF *Schistocerca* HOPPERS ON VARIOUS STEROIDS

Exp.	Steroid in diet	Mean wt. (mg) and numbers of hoppers alive at these times after hatching			Adults		
		6 days	12 days	26 days	♂♂	♀♀	Aborts
1	Cholesterol (0·28 mg/g diet)	65 (16)	196 (3)	572 (2)	0	0	1
	Cholesterol (1·4 mg/g diet)	93 (20)	253 (10)	1365 (8)	5	3	
	β-Sitosterol (1·4 mg/g diet)	112 (16)	334 (12)	1568 (9)	5	4	
	Stigmasteryl acetate (1·4 mg/g diet)	56 (18)	124 (7)	All dead	0	0	
	Ergosterol (1·4 mg/g diet)	40 (15)	All dead		0	0	
	Lanosterol (1·4 mg/g diet)	64 (17)	133 (9)	All dead	0	0	
	Squalene (2·7 mg/g)	44 (7)	88 (3)	All dead	0	0	
	Calciferol (1·4 mg/g)	36 (9)	51 (2)	All dead	0	0	
	Testosterone (0·7 mg/g)	39 (16)	75 (6)	All dead	0	0	
	Oestrone (1·4 mg/g)	39 (17)	71 (6)	All dead	0	0	
2	Cholic acid (1·4 mg/g diet)	62 (6)	116 (2)	All dead	0	0	
	Cholesterol (1·4 mg/g diet)	98 (20)	294 (19)	1526 (13)	5	8	
	β-Sitosterol (1·4 mg/g diet)	102 (14)	300 (14)	1588 (8)	4	4	
	7-Hydroxycholesterol dibenzoate (1·4 mg/g diet)	84 (9)	138 (2)	All dead	0	0	
	Cholestan-3β-ol (1·4 mg/g diet)	87 (10)	261 (11)	1308 (9)	8	1	
	7-Dehydrocholesterol (1·4 mg/g diet)	50 (8)	58 (2)	All dead	0	0	
	Cholestenone (1·4 mg/g diet)	37 (1)	All dead		0	0	
	7-Oxocholesteryl acetate (1·4 mg/g diet)	54 (14)	123 (5)	All dead	0	0	
3	No sterol	51 (20)	110 (3)	All dead	0	0	
	Cholesterol (1·4 mg/g)	72 (14)	274 (10)	1453 (6)	1	5	
	Cholesteryl acetate (1·4 mg/g)	93 (15)	293 (10)	1212 (6)	4	–	3
4	Cholesterol (0·28 mg/g)	47 (11)	98 (7)	417 (1)	0	0	
	Cholesteryl acetate (1·4 mg/g)	77 (16)	244 (10)	1429 (9)	5	4	
	Stigmasteryl acetate (1·4 mg/g)	49 (6)	All dead		0	0	
	Ergosterol (1·4 mg/g)	55 (8)	105 (7)	383 (3)	0	0	
	Cholestan-3 β-ol (1·4 mg/g)	86 (16)	217 (14)	1317 (6)	3	3	
	7-Dehydrocholesterol (1·4 mg/g)	59 (14)	99 (3)	All dead	0	0	
	Cholestenone (1·4 mg/g)	58 (7)	121 (1)	All dead	0	0	
	7-Oxocholesteryl acetate (1·4 mg/g)	61 (10)	120 (1)	All dead	0	0	

acetate and cholesterol (Exp. 3). The acetate was clearly utilized as well as was cholesterol.

A final experiment served to check the results obtained with the more crucial substances already tested (Exp. 4). Growth occurred only with cholesteryl acetate and dihydrocholesterol.

TABLE 3—GROWTH OF GROUPS OF *Locusta* HOPPERS ON VARIOUS STEROIDS

Exp.	Steroid in diet	Mean wt. (mg) and number of hoppers alive at these times after hatching			Adults		
		6 days	12 days	26 days	♂♂	♀♀	Aborts
1	Cholesterol (0·28 mg/g)	39 (15)	All dead		0	0	
	Cholesterol (1·4 mg/g)	66 (13)	182 (13)	1012 (5)	2	3	
	β-Sitosterol (1·4 mg/g)	75 (18)	208 (14)	1100 (10)	4	6	
	Stigmasteryl acetate (1·4 mg/g)	29 (5)	All dead		0	0	
	Ergosterol (1·4 mg/g)	27 (6)	All dead		0	0	
	Lanosterol (1·4 mg/g)	27 (9)	All dead		0	0	
	Squalene (2·1 mg/g)	36 (1)	All dead		0	0	
	Calciferol (1·4 mg/g)	All dead					
	Testosterone (0·7 mg/g)	All dead			0	0	
	Oestrone (1·4 mg/g)	34 (5)	All dead		0	0	
2	Cholic acid (1·4 mg/g)	38 (6)	All dead		0	0	
	Cholesterol (1·4 mg/g)	71 (17)	189 (9)	745 (5)	4	1	
	β-Sitosterol (1·4 mg/g)	75 (16)	204 (15)	768 (4)	3	1	
	7-Hydroxycholesterol dibenzoate (1·4 mg/g)	27 (2)	All dead		0	0	
	Cholestan-3β-ol (1·4 mg/g diet)	65 (19)	164 (14)	819 (9)	4	3	1
	7-Dehydrocholesterol (1·4 mg/g diet)	36 (8)	All dead		0	0	
	Cholestenone (1·4 mg/g diet)	30 (3)	All dead		0	0	
	7-Oxocholesteryl acetate (1·4 mg/g diet)	36 (3)	All dead		0	0	
3	No sterol	46 (13)	All dead		0	0	
	Cholesterol (1·4 mg/g)	66 (10)	201 (10)	1007 (3)	1	2	
	Cholesteryl acetate (1·4 mg/g)	66 (10)	213 (10)	1104 (6)	3	1	1
4	Cholesterol (0·28 mg/g)	40 (9)	53 (4)	All dead	0	0	
	Cholesteryl acetate (1·4 mg/g)	62 (15)	162 (11)	809 (6)	2	3	1
	Stigmasteryl acetate (1·4 mg/g)	37 (8)	All dead		0	0	
	Ergosterol (1·4 mg/g)	38 (17)	52 (5)	All dead	0	0	
	Cholestan-3β-ol (1·4 mg/g diet)	58 (15)	144 (11)	780 (7)	2	3	1
	7-Dehydrocholesterol (1·4 mg/g diet)	35 (7)	All dead		0	0	
	Cholestenone (1·4 mg/g diet)	31 (15)	All dead		0	0	
	7-Oxocholesteryl acetate (1·4 mg/g diet)	37 (7)	All dead		0	0	

DISCUSSION

It has generally been found that steroids differing extensively from cholesterol are not utilized by insects. This appears so with locusts; growth with oestrone, testosterone, cholic acid, calciferol, and lanosterol was no better than in the

absence of steroids. The negative results obtained with squalene and lanosterol show that these substances which may be intermediates in the biosynthesis of cholesterol from acetate in mammals (SCHNEIDER *et al.*, 1957) cannot be utilized by locusts. Squalene could not replace cholesterol in the diet of *Dermestes*, though this insect was able to synthesize it from acetate (BLOCH *et al.*, 1956).

Of the sterols and derivatives tested, only cholesterol, β-sitosterol, and dihydrocholesterol allowed good growth. Utilization of the two last named-substances show that substitution of an ethyl group at position 24 of cholesterol, or saturation of the nucleus, are without detrimental effect. The negative results obtained with cholestenone, 7-oxo-cholesteryl acetate, and 7-hydroxycholesterol dibenzoate were anticipated, for a hydroxyl group, free or esterified, at position 3 has been essential for utilization by all insects studied, and an additional hydroxyl, or a ketone group at position 7 has always rendered the compound inactive (LEVINSON, 1955).

The negative result with 7-hydrocholesterol shows that for locusts a double bond at position 7 of cholesterol inhibits utilization. If, as the experiment with cholesteryl acetate suggests, esterification of the 3-hydroxyl group with acetate is no bar to utilization, the failure of stigmasteryl acetate indicates that a double bond at position 22 renders the substance inactive, as this is the only point of difference from β-sitosterol. It should be borne in mind, however, that although acetates have generally been found as effective as the unmodified sterol, esterification with acetate rendered ergosterol non-utilizable to *Blattella* (NOLAND, 1954a). Failure to utilize ergosterol may be understood in relation to inhibition by these double bonds, for it has them both.

The negative results obtained with 7-hydrocholesterol, ergosterol, and stigmasterol are most surprising. The former is utilized to some extent by all insects previously studied, and there is some indication that it occurs in the tissues of insects given dietary sitosterols (BECK and KAPADIA, 1957). Hitherto only the strictly zoophagous species *Dermestes* and *Attagenus* have failed to utilize ergosterol (LEVINSON, 1955), and stigmasterol or its acetate has allowed some growth in all insects with which it has been tested, being superior to cholesterol for the phytophagous species *Callosabruchus* (ISHII, 1951) and *Pectinophora* (VANDERZANT and REISER, 1956).

Apparently quite minor structural characters determine what sterols are utilized and differentiate their availability to various species. In view of this and the probable dependence of absorption of sterols on esterification in the gut, a process for which the configuration at position 3 is crucial (NOLAND, 1954b), it has been suggested that specificity in sterol requirements may be due to highly specific esterase systems governing absorption (NOLAND, 1954a). Such a system, able to hydrolyse and esterify cholesterol, Δ^7-cholesterol, and 7-dehydrocholesterol, though not ergosterol or stigmasterol, has been demonstrated in gut tissues of *Periplaneta* (CASIDA *et al.*, 1957). Unfortunately no data on the nutritional value of these sterols are available for this cockroach; the closely

related *Blattella* utilizes all these substances with the exception of ergosterol as the acetate (NOLAND, 1954a).

It seems clear that patterns of sterol utilization in insects are too various to permit of simple division into zoophagous and phytophagous types. It is true that all phytophagous (meaning non-zoophagous) species tested have utilized cholestan-3β-ol (LEVINSON and BERGMAN, 1957) and locusts share this ability. However, the inability of locusts to utilize 7-hydrocholesterol marks them off from all insects hitherto examined, and this, together with failure as in the zoophagous species to utilize ergosterol, confuses the hypothesis.

This does not detract from the value of the idea that sterol utilization is related to natural diet; evidence for such a relationship is abundant and the data for locusts suggests rather narrower specificity than has hitherto been contemplated. Further information on other plant-eating insects and on other phytosterols would be of the utmost value and might contribute to an understanding of the general problem of food specificity in oligophagous insects.

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SUMMARY

1. Using synthetic diets the effect of various concentrations of cholesterol on the growth of *Schistocerca gregaria* (Forsk.) and *Locusta migratoria* L. was determined. With both species growth was optimal with 0·84 mg cholesterol/g diet, and with 0·28 mg/g or less little growth occurred.

2. The utilizability of various steroids was determined by comparing growth on diets containing them in place of cholesterol with growth on diets containing cholesterol or no sterol.

3. Of the substances tested only cholesterol, cholesteryl acetate, β-sitosterol, and dihydrocholesterol (cholestan-3β-ol) were utilized. The following were unable to replace cholesterol: calciferol, cholic acid, testosterone, oestrone, lanosterol, squalene, ergosterol, stigmasteryl acetate, 7-dehydrocholesterol, 7-oxocholesteryl acetate, and cholestenone.

4. The effect of sterol structure on utilizability, and the special features shown by locusts in this respect, are discussed in relation to work on other insects.

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COLD HARDINESS IN THE EUROPEAN CORN BORER, *PYRAUSTA NUBILALIS* (HÜBN.)*

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Abstract—The relationships between undercooling capacity, tissue freezing, and survival at subzero temperatures were studied in mature larvae of the European corn borer, *Pyrausta nubilalis* (Hübn.). Undercooling temperatures were determined by means of a thermistor-thermometer. Larval survival was measured at −10, −15, and −20°C. It was found that the undercooling temperatures of the larvae were not a reliable indication of their ability to survive under subzero conditions.

Cold-hardening of borer larvae in the field began in August and continued to increase until late November. Summer larvae in diapause succumbed rapidly to low temperatures. Cold-hardy winter larvae, on the other hand, survived up to 3 months at −20°C, when in the absence of contact moisture. Chilling in the presence of contact moisture caused freezing of the larvae and reduced their ability to survive cold conditions. Despite the formation of ice in their tissues, fully cold-hardy borers survived several weeks while frozen at −20°C.

The undercooling temperatures of cold-susceptible larvae were lowered by chilling and desiccation, but such treatment did not significantly enhance their ability to survive subzero conditions. Cold hardiness was destroyed by incubating cold-hardy larvae at 30°C, either with or without contact moisture.

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INTRODUCTION

THE European corn borer survives the winter in diapause as mature larvae in various parts of old corn stalks. Diapause induction is controlled mainly by temperature and photoperiod during the larval stage (MUTCHMOR and BECKEL, 1959; BECK and HANEC, 1960). Diapause is an arrest of development and, from a teleological standpoint, provides the borer with a means of surviving a period in which there are no suitable food plants available. This not only ensures a synchronization of the insect's life cycle with that of its host plant, but also constitutes an important part of its ability to survive the winter. But a state of diapause does not, in itself, enable the borer to survive at winter temperatures. It is apparent that other physiological changes occur which result in greater endurance of the larva under freezing conditions. The increasing ability of an insect to survive subzero temperatures is termed cold-hardening; the degree of cold-hardening which an insect undergoes is expressed as its cold hardiness.

According to current concepts of winter survival of insects, there are at least two types of cold hardiness possessed by insects. One type is a resistance to freezing of the body fluids, or supercooling; the insect may be supercooled many

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degrees below its true freezing point without ice formation in its tissues. Once frozen, the insect does not survive. The extent to which it can be chilled before freezing occurs has been assumed to be an expression of its cold hardiness; i.e. the undercooling temperature may serve as an index of cold hardiness. It has been postulated that the ability to supercool is the only low temperature protection that most hibernating insects possess (SALT, 1958c). The extent of supercooling that an insect can undergo is not a fixed property, but apparently depends on cold-conditioning, time and temperature of chilling, the nature and type of nucleating agents within the insect (SALT, 1958a), and probably other factors as yet unknown. SALT (1953) postulated that food particles present in the gut tract could act as nucleating agents and lower the cold resistance (as expressed by the undercooling temperature) of feeding insects. He stated that cold hardiness of insects in diapause occurs only because they do not feed (SALT, 1958b). However, there has been some doubt as to the validity of the assumption that the undercooling temperature can be used as a reliable index of cold hardiness (BARNES and HODSON, 1956), unless the factors which can modify the degree of supercooling are also considered, particularly the time–temperature complex during the determination of undercooling points (SALT, 1950, 1953). Despite the general use of the undercooling temperature as an index of cold hardiness in insects, there is little experimental evidence to show that such an assumption is valid, and there exists a gap in the understanding of the relationship between the undercooling points as obtained by laboratory methods and the survival of insects at subzero temperatures.

The European corn borer is an example of an insect that can tolerate the formation of ice crystals in its tissues and still survive. This second type of cold resistance has been largely neglected, and little is known concerning the adaptation and cold resistance of insects in this group. The borer provides another interesting aspect in the study of cold hardiness because, in many instances, the overwintering location is very wet and the larva may be completely frozen and encased in ice. Earlier authors (HODSON, 1937; SACCHAROV, 1928; BARBER and DICKE, 1940) found that survival of insects was markedly reduced under wet conditions.

Since the first generation of borer larvae cease feeding and enter diapause in August, they are not initially cold-hardy, but as the season progresses they acquire sufficient cold hardiness to survive several months in the frozen state. BARNES and HODSON (1956) found that mature borer larvae were very cold-susceptible during late summer but by mid-October could survive freezing following maximum undercooling. The authors felt that during the winter, the insect's undercooling temperature was of little significance as an indicator of cold hardiness because cold-hardy larvae survived freezing for at least 3 weeks in the laboratory and the entire winter in the field. On the other hand, DITMAN *et al.* (1942, 1943) working with the mature borer larvae among other insects, concluded that 'the comparative cold hardiness of insects may best be ascertained directly by undercooling point determinations'. Neither of the authors compared the survival of borer larvae under controlled subzero temperatures with the undercooling temperatures as obtained in the laboratory.

The following aspects of cold hardiness in the mature European corn borer larvae were investigated: (a) whether the environmental factors responsible for diapause induction in the larvae in any way contributed to the insect's cold hardiness, (b) the cold-hardening of the larvae as the season progressed and the response of the insect to cold during this period, and (c) the effect of contact moisture during the chilling period on the survival of the larva.

MATERIALS AND METHODS

The majority of the larvae used in this study were dissected out of field corn stalks during the summer and early fall of 1958. During late fall several thousand infested corn stalks were cut and stored outside and the larvae were removed from these stalks as needed during the course of this work. After the larvae were brought into the laboratory, they were warmed and dried before they were placed in chilling cabinets to determine survival. For certain aspects of this work, the undercooling temperatures of larvae were obtained. The undercooling temperature (U.T.) was defined as the lowest temperature to which the larvae could be chilled before freezing of the tissues commenced. The undercooling temperatures were determined by means of a previously untried instrument, the thermistor thermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). The apparatus consisted of a temperature-sensitive element set inside the distal end of a twenty-two gauge probe. The probe was connected to a transistorized temperature recording unit on which the surface temperature of the insect could be directly read. The range of the instrument was from 10 to $-40°C$. The accuracy was within $\pm 1°C$ at the extreme ends of the scale and probably less than $\pm 0{\cdot}5°C$ through the range of temperatures that were used in this work. This instrument reacts extremely rapidly to sudden temperature changes, the 'time constant' being 0·6 sec. The term 'time constant' is used to refer to the time required for a given probe to indicate 63 per cent of a sudden temperature change to which it is subjected. The U.T. determinations were made at temperatures of -30 to $-35°C$.

Since this method of determining the U.T. of insects had not been previously used, it was necessary to determine its range of accuracy. It was assumed that a fair test of the method would be to determine whether the undercooling temperature could be consistently reproduced for any particular larva (SALT, 1936). Five consecutive U.T. determinations were made for each of nine larvae. The insects and probe were dried and warmed for 10 min between the determinations. The time of chilling from 10°C to the temperature at which the U.T. occurred, varied from 6 to 8 min. The larvae did not show any symptoms of injury due to the repeated chilling and warming.

Analyses of variance showed no significant differences among the five consecutive undercooling points obtained for each larva. However, there was a highly significant difference among the average undercooling temperatures of the different individuals. On the assumption that the reproducibility of results constituted a measure of precision, the procedure was considered fully adequate for this type of study.

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The water content of the larvae was obtained by drying the insects at 85–90°C for 48 hr. Fat content was determined by extracting the dried larvae with ether for 8 hr in a Soxhlet extractor. Fat content was expressed as per cent of ether-solubles per 100 mg of dry weight.

Larval mortality at subzero temperatures was determined by placing the insects in individual numbered vials at −10, −15, and −20°C. The variation of temperature inside the cold cabinets did not exceed ±1°C from the desired setting. To determine mortality, the larvae were removed periodically from the cold, warmed for about 24 hr, and examined; those that failed to respond to gentle prodding were considered dead. The survivors were returned to the freezers. The majority of the larvae that died in the cold became discoloured very shortly after being warmed and were easily distinguished from the living. However, some larvae were 'border-line' cases, because on being warmed they became extremely flaccid and somewhat paralysed. These larvae were incapable of locomotion and merely moved their prolegs, mouth parts, or displayed feeble contractions of the abdomen. Such larvae usually died upon a further short period of chilling and very few recovered even in a warm incubator. The larvae in this category were considered alive until such time as movement ceased entirely.

It must be emphasized here that this chilling régime was not intended to provide data which would show the survival of larvae under conditions which they would experience in the field or even at constant low temperatures until they died. The periodic thawing and chilling was undoubtedly harmful to the larvae. However, since all the larvae were exposed to the same chilling and thawing cycle, it is assumed that this procedure would indicate the *relative* cold hardiness of the various groups of larvae that were treated in this manner.

RESULTS

I. *Survival of mature non-diapause European corn borer larvae at subzero temperatures*

The larvae for this study were reared at 30°C in darkness; none entered diapause under these rearing conditions. The U.T. was determined after feeding ceased and prior to the prepupal stage. After the U.T. was determined, the larvae were placed in individual dry vials at −10°C to determine cold mortality. Fifty per cent mortality occurred in less than 3 days, complete mortality in about 6 days. All the larvae were severely injured by the cold after only about 24 hr of chilling. For all practical purposes, these larvae could have been considered dead, because similarly damaged larvae placed in warm incubators invariably failed to recover, although some survived for several days.

II. *The relationship between the U.T. and survival of borer larvae at subzero temperatures*

The larvae were reared at 28°C under a 10·5 hr photoperiod; all the mature larvae entered diapause under these conditions. Samples of fifteen larvae were removed from the rearing incubator at regular intervals after feeding had ceased. The U.T. was determined, and the insects were chilled dry at −10°C. 'Dry' chilling

consisted of placing the larvae in individual dry vials in the low temperature cabinets; conversely, for 'wet' chilling, the larvae were placed in vials containing very wet absorbent paper. In the latter situation, the larvae always had free water droplets on their surfaces before being placed in the cold.

The larvae were removed periodically from the cold cabinets, warmed for 24 hr, and mortality was noted; the survivors were then returned to the cold for further chilling. Additional samples of ten larvae were removed from the rearing incubator at the same time as the sample to be chilled, and water and fat content was determined. It was expected that the water content of the mature larvae in dry vials in the incubator would drop slowly, causing the U.T. also to fall. The problem was to determine what effect the decline in U.T. might have on cold survival.

TABLE 1—THE RELATIONSHIP OF THE U.T. AND SURVIVAL OF LABORATORY-REARED DIAPAUSE BORER LARVAE AT −10°C (DRY CHILLED).

Days after feeding ceased	Per cent water	Per cent fat	Average U.T.* (− °C)	Average R.T. (− °C)	T_{50D}† (days)
2	66·4	44·1	10·5 ± 2·1	0·38	4
13	55·8	41·2	13·3 ± 2·3	0·80	4
25	52·0	39·1	17·8 ± 2·3	2·34	5
39	48·2	44·4	18·6 ± 1·1	3·70	5

* L.S.D. for U.T. is 2·63 at the 1 per cent level.

† T_{50D} is the exposure time in days required for 50 per cent mortality.

The undercooling temperature dropped from −10·5 to −18·6°C during 39 days (Table 1), but mortality at −10°C was virtually unchanged. From 4 to 5 days were required for 50 per cent mortality and from 10 to 12 days for complete mortality (Fig. 1). Obviously, the cold hardiness of these larvae cannot be compared on the basis of undercooling points. Actual mortality or survival data at subzero temperatures must be obtained before comparisons of cold hardiness can be made. Observations on the larvae immediately after they were removed from the freezer showed that the insects did not freeze at −10°C, except for one larva in the first group, and it died on thawing.

III. *The effect of heat and contact moisture on the undercooling temperature and survival of cold-hardy European corn borer larvae*

BARNES and HODSON (1956) found that at moderately high temperatures and in the absence of free moisture, the undercooling point of European corn borer larvae rose very little. The authors concluded from further experimental data that it was 'the effect of temperature and moisture acting together which brings about the rapid and marked rise of the undercooling level of cold-hardy, fifth instar corn borer larvae'. LOZINA-LOZINSKII (1935) reported that European borer larvae

froze more easily after they were held for some time at 20°C. It was of interest to determine the effect of wet and dry incubation on the U.T. and survival of cold-hardy borer larvae. A large sample of field larvae was collected on 23 December 1958. Their average U.T. was −16·6°C, the average water and fat contents were 56·2 and 36·9 per cent respectively. A group of thirty-five larvae was placed in dry vials at 30°C, a comparable group was also placed at 30°C, but supplied with contact water. After 10 days of incubation all the larvae were reweighed, the U.T. and R.T. redetermined, and samples were taken for determination of fat and water content. To obtain mortality data, the larvae were dry-chilled at −20°C. The results are summarized in Table 2.

TABLE 2—THE EFFECT OF HIGH TEMPERATURE AND CONTACT MOISTURE ON THE U.T. AND SURVIVAL OF COLD-HARDY CORN BORER LARVAE

Treatment	Average U.T.* (− °C)	Average R.T. (− °C)	Per cent water	Per cent fat	T_{50D} (−20°C) (days)
Field larvae	16·6	4·67	56·2	36·9	57 dry 32 wet
10 days at 30°C, dry	15·0	1·80	56·1	43·1	4 dry
10 days at 30°C, wet	12·8	0·60	68·9	42·4	1 dry

* L.S.D. for U.T. is 2·32 at 1 per cent level.

The results showed a significant rise in the U.T. of only the wet-warmed larvae; the undercooling points of the dry-warmed and field larvae were essentially similar, as BARNES and HODSON (1956) found in their work. On the assumption that the U.T. is an index of cold hardiness, these results may be interpreted as implying that the dry, warm treatment was insufficient to destroy the cold resistance of the larvae. Actually, this is untenable because as the mortality data show, the 'dry' and 'wet' incubated larvae were essentially equally susceptible to cold at −20°C. The wet-warmed larvae were surface dried before being placed at −20°C, but despite this precaution all the larvae were frozen after 24 hr chilling. This was a considerable departure from the behaviour of the dry-warmed larvae, of which only about one-third froze during the first day of chilling.

The larvae that were incubated with contact water did not all gain weight; a few lost up to 10 per cent of their original weight. Of those that gained weight, the moisture gain was from 2·9 to 25·6 per cent of their original weight. A correlation was calculated between the per cent weight increase and the U.T. after the incubation period. The correlation factor (r) obtained was −0·388, ($r_{(0\cdot57)}$ is 0·361). This indicates a weak negative correlation between the weight gained and the U.T. It was not possible to determine the relation between the amount of water picked up by individual larvae and duration of survival because of the very short survival period of the whole test sample.

IV. *Cold hardiness of mature European corn borer larvae collected from the field during late summer, fall, and winter of* 1958

The larvae for this study were collected periodically from late August through to the end of December 1958, from a cornfield about 30 miles north of Madison, Wisconsin. The larvae were in diapause and the majority were from the first generation. The second generation began so late in the season that it is doubtful if many larvae matured sufficiently to overwinter successfully. Only fully grown, mature larvae were used in this study.

The purpose of this investigation was to follow the cold mortality of field larvae as the season progressed. Since borer larvae frequently overwinter in wet locations inside the corn stalk, survival under both wet and dry chilling conditions was determined. During each collection period, about 200 larvae were used.

Mortality curves were made for each group of larvae exposed (Figs. 1–6). The number of larvae that froze in the dry chilling situations was also observed. It should be mentioned here that the term 'froze' does not imply death but is used to denote those larvae which were frozen in the subzero cabinets. It differs from the term 'flaccid' which was used to describe the soft non-frozen larvae.

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DISCUSSION

The data obtained in this study have disclosed some important features of the response of the European corn borer to subzero temperatures. It was found that cold-hardy larvae could survive periods of being in a solid frozen state. Non-cold-hardy larvae, on the other hand, were unable to survive more than a few days of subzero temperatures, even though the temperatures employed were such that the larvae remained in a flaccid, unfrozen condition. It was apparent, therefore (Tables 1 and 2), that the supercooling capacity of the insect could not be interpreted as a measurement of its cold hardiness. Although there was a significant lowering of undercooling temperatures accompanying desiccation of larvae in diapause, survival at $-10°C$ was unchanged (Table 1). Incubating cold-hardy larvae at 30°C for several days tended to destroy their ability to survive prolonged exposure to subzero temperatures. However, their undercooling temperatures were raised only in the presence of contact moisture (Table 2). The ability of European corn borer larvae to supercool appeared to be in no way related to the duration of survival under subzero conditions.

Observations on cold-hardy field grown larvae showed that the majority of borers did not actually freeze under dry-cold conditions. There did not appear to be a relationship between the duration of survival and a frozen or flaccid state. Flaccid larvae tended to survive no longer than frozen ones, at a given temperature. This effect was interpreted as indicating that mortality was caused by some factor other than ice formation in the general body tissues.

Undercooling temperature may be an adequate criterion of cold hardiness in insects whose survival depends on their ability to supercool, and which die when

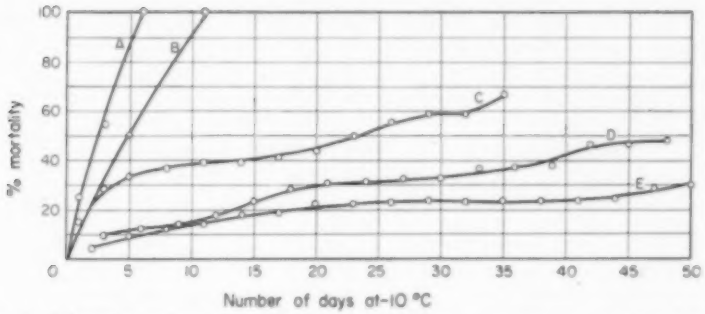

FIG. 1. Mortality curves of different groups of European corn borer larvae at -10°C, chilled dry. (A) Non-diapausing, non-cold-hardy larvae; (B) incubator-reared diapause non-cold-hardy larvae; (C) field larvae collected on 29 August; (D) field larvae collected on 10 September; (E) field larvae collected on 13–14 October.

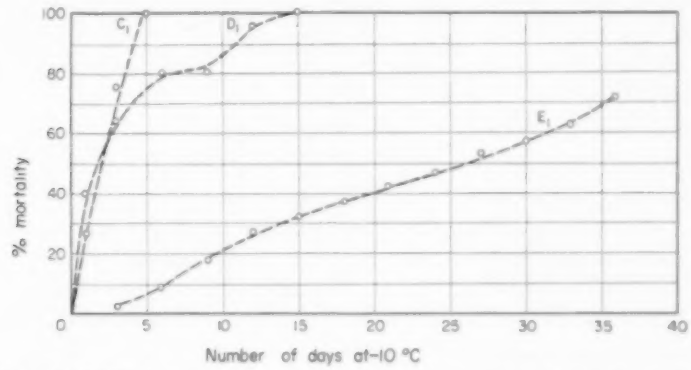

FIG. 2. Mortality curves of different groups of European corn borer larvae at −10°C, chilled wet. (C_1, D_1, E_1) Diapausing field larvae collected on 29 August, 10 September, and 13–14 October 1958, respectively.

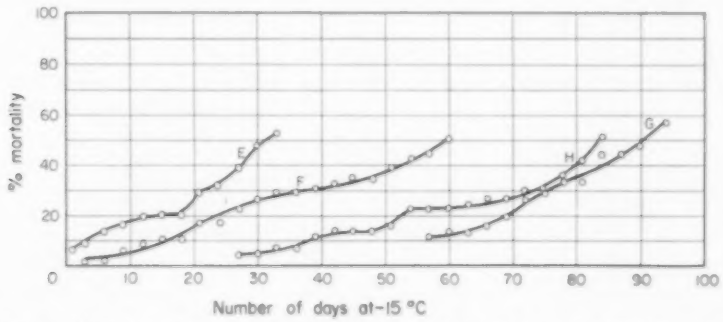

FIG. 3. Mortality curves of different groups of field borer larvae chilled dry at −15°C. (E) Larvae collected on 13–14 October; (F) larvae collected on 9–10 November; (G) larvae collected on 28–29 November; (H) larvae collected on 23 December 1958.

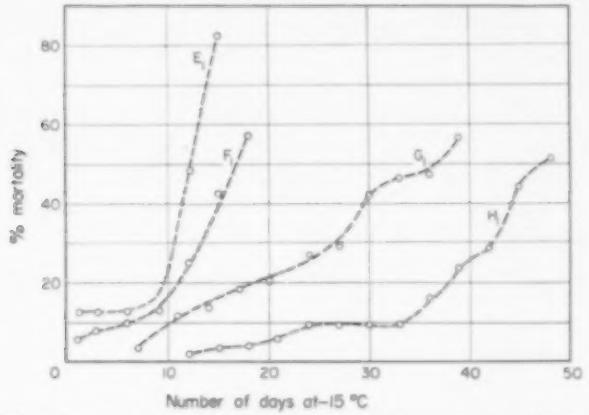

FIG. 4. Mortality curves of different groups of field borer larvae, chilled wet at −15°C. (E_1, F_1, G_1, H_1) Larvae collected on 13–14 October, 9–10 November, 28–29 November, and 23 December 1958, respectively.

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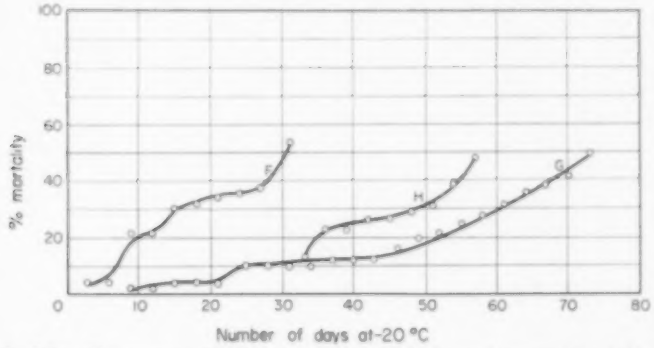

FIG. 5. Mortality curves of different groups of field borer larvae, chilled dry at −20°C. (F, G, H) Larvae collected on 9–10 November, 28–29 November, and 23 December 1958, respectively.

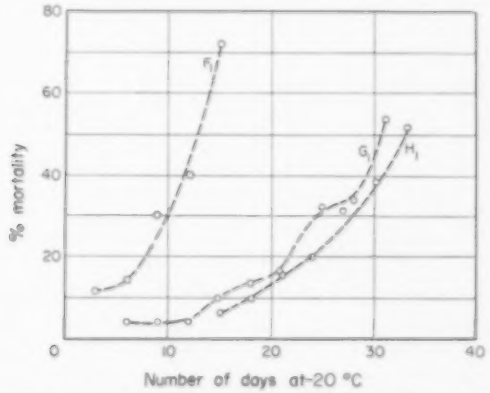

FIG. 6. Mortality curves of different groups of field borer larvae, chilled wet at −20°C. (F_1, G_1, H_1) Larvae collected on 9–10 November, 28–29 November, and 23 December 1958, respectively.

ice formation occurs. Even with this type of insect, undercooling temperature may be an inadequate basis for the estimation of cold hardiness. SALT (1953) compared 'cold hardiness' of feeding and non-feeding stages of *Agrotis* and *Ephestia* on the basis of their undercooling temperatures. On this basis, he concluded that the non-feeding stages were more cold hardy than the feeding stages. The ability of the insects to survive prolonged exposure to freezing (subzero) conditions was not demonstrated. Cold hardiness is the ability to survive an arbitrary period of days or weeks of an arbitrary subzero temperature, not its capacity for supercooling over a period of a few minutes. A correlation between these two phenomena has been assumed but never demonstrated under adequate experimental conditions.

TABLE 3—THE T_{50D} OF EUROPEAN CORN BORER LARVAE AT DIFFERENT SUBZERO TEMPERATURES. THE LARVAE WERE COLLECTED AT INTERVALS FROM THE FIELD DURING AUGUST TO DECEMBER 1958

Collection date	Days to 50 per cent mortality (T_{50D})					
	−10°C		−15°C		−20°C	
	Dry	Wet	Dry	Wet	Dry	Wet
28 August	23	1–3	1	—	1	—
10–12 September	56	1–3	1	—	1	—
13 October	50	27	33	13	1	—
9–10 November	—	—	60	17	30	13
28–29 November	—	—	90	37	73	31
23–26 December	—	—	84	48	57	33

SALT (1958b) suggested that insects in diapause are 'cold-hardy because they do not feed', presumably on the assumption that food remnants in the gut tract act as nucleating agents (SALT, 1953). The results of the present study show that both laboratory-reared larvae (Fig. 1 B) and larvae field-collected in August and September (Fig. 2 C_1, D_1) exhibited little or no cold hardiness even though they were in diapause and their gut tracts were presumably empty (BECK *et al.*, 1950). Diapause may serve as a period during which the insect undergoes a physiological cold hardening, but it is apparent that neither the conditions inducing the arrested development, nor diapause itself produce cold hardiness.

An insect may be quite cold-hardy at one temperature, but very poorly cold resistant to a lower temperature. SALT (1958a) found that *Cephus cinctus* larvae required from 2100 to 2800 hr to reach 50 per cent mortality at −15°C, but less than 0·15 hr at −26°C. Our results with the European corn borer showed a similar, but less striking, relationship. It was also noted that the mortality induced by a given low temperature declined as the seasons advanced (Figs. 1–6, Table 3). For example, August-collected borer larvae died in less than 1 day at −15°C under dry conditions. By late November, the cold hardiness of field-collected borers had

increased to such an extent that about 90 days were required for 50 per cent mortality. This type of changing reaction was observed at all subzero temperatures employed in the study.

Corn-borer larvae chilled under dry conditions survived at least twice as long as those chilled at the same temperature in the presence of contact moisture (Table 3). Borers collected during the late summer and early fall were much more sensitive to wet chilling than were the hardier borers collected during the winter. In all experiments, larvae chilled with contact moisture quickly froze upon being placed in the low temperature cabinets. The higher mortality of the wet-chilled larvae may have been the result of their being subjected to numerous freezing–thawing cycles, as they were checked for mortality and then returned to the freezers. The mortality data from the dry-chilled groups of larvae indicated no apparent relationship between freezing and survival.

The results of our cold-survival experiments might suggest that natural mortality of borers under field conditions should be very high during the winter months. No attempt was made to duplicate winter weather conditions, and the high mortality observed in the laboratory experiments carries no implications concerning field survival. The chilling and thawing cycles necessary for mortality counts may have been more deleterious than a constant low temperature.

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EFFECTS OF DIETARY GLUCOSE ON HAEMOLYMPH CARBOHYDRATES OF *AGRIA AFFINIS* (FALL.)

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Abstract—The blood of third-instar larvae of the dipterous parasite, *Agria affinis* (Fall.) [= *Pseudosarcophaga affinis* (Fall.)], reared on chemically defined diets under aseptic conditions was analysed for carbohydrates. The concentration of total carbohydrate is high early in the instar and significant decreases occur at about the fourth and eighth day. It is suggested that the first decrease may be due to cessation of procuticle secretion and the second to cessation of feeding prior to pupation. Blood carbohydrates in mature larvae are not changed by diets containing from 0 to 1 per cent glucose but they can be increased, though not changed qualitatively, by increasing the glucose content of the diet to 2 per cent. Immature larvae, however, appear to be unable to synthesize carbohydrate at a rate sufficient to maintain the high level of carbohydrate in their blood. Quantitatively, glucose comprises at least 80 per cent of the total carbohydrate. Fructose is the next most abundant (5–6 per cent) and there are small amounts of trehalose (1–2 per cent), pentoses, probably arabinose and ribose (1–2 per cent) mannose, and unidentified polysaccharides (3–4 per cent). This is in contrast to analyses of blood of several other species of insect in which trehalose comprises some 90 per cent of the total carbohydrate.

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INTRODUCTION

THE present study determined qualitatively and quantitatively the sugars present in the blood of larvae of *Agria affinis* (Fall.) [= *Pseudosarcophaga affinis* (Fall.)] as a prelude to a study of the digestion and absorption of sugars by these larvae. *A. affinis* is particularly useful for such studies because it can be reared on a chemically defined diet under sterile conditions. This permits precise qualitative and considerable quantitative control of the materials ingested. Because of the small size of the larvae, the study was limited to the last (third) larval instar.

MATERIAL AND METHODS

Agria affinis larvae were reared on chemically defined diets with sterile techniques as described by HOUSE and BARLOW (1956).

Carbohydrates were determined using anthrone. Several anthrone reagents were tried (FALES, 1951; FAIRBAIRN, 1953; MOKRASCH, 1954; CARROLL *et al.*, 1956). The method of FALES was finally adopted because of its simplicity. Three millilitres of reagent were mixed with 0·3 ml of trichloracetic acid (TCA) (10 per cent) or alcohol (70 per cent) filtrate of blood. Standard solutions of glucose in 10 per cent TCA were analysed with each batch of blood samples. Twenty microlitres of blood were used for each sample.

For chemical analyses blood was taken from larvae coddled for 2 min at 60°C. The samples of blood taken from each larva were routinely limited to 5 μl although a larger volume could sometimes be obtained. Larvae from which 5 μl of blood could not be obtained were discarded. Thus the analysis of the pooled sample gave values which were true averages of the individual samples.

Chromatograms of blood sugars were made on strips of Whatman No. 1 filter paper 9 in. wide and 18 in. long. The preparation of blood for chromatography was similar to that used by WYATT and KALF (1957). Blood from approximately 250 larvae, collected as described above but regardless of the volume contributed by each larva, was put under mineral oil; approximately 2 ml of blood were obtained. This was centrifuged and the clear plasma decanted. As in *Phormia* and *Calliphora* the cells occupy only about 3 per cent of the total volume (BUCK, 1953), thus there was no detectable difference in carbohydrate content between whole blood and plasma. Triplicate 20 μl samples were taken for chemical analysis for total, alcohol (70 per cent) soluble, and un-ionized carbohydrate. Samples of 0·2 ml were taken for chromatography. These were made up to 70 per cent alcohol, heated to 75°C for 5 min, centrifuged, and decanted through an ion exchange column; the residue was washed four times with 0·2 ml of 70 per cent alcohol (heating, centrifuging, and decanting as above). Each washing was added to the column when most of the preceding solution had entered the resin bed so that the resins were well washed.

Ion exchange columns about 1·5 in. long were made by layering equal volumes of Dowex 50–X12, 200/400 mesh, H-form and Dowex 2-X10, 200/400 mesh, formate from slurries in 70 per cent alcohol on a glass wool plug in a 1 ml tuberculin syringe. A plastic rack was made to hold the filter paper between two plates with 1 in. diameter holes at the origin of the chromatograms. The tuberculin syringes, fitted with No. 30 needles tipped with polyethylene tubing drawn to a taper were suspended from a bridge above the paper by a ring of heavy walled rubber tubing. The end of the polyethylene tubing touched the paper at the origin of the chromatogram. The diameter of the spot produced was kept to about 1 cm by varying the flow of a stream of hot air directed at the underside of the origin of the chromatogram. The rate of flow of solution was increased if necessary by shortening the taper to increase the size of the opening.

With this apparatus, several samples of haemolymph could be extracted and the total of about 1·6 ml of alcohol extract from each could be de-ionized and spotted on the paper in 3–4 hr. Across each chromatogram was spotted first, a solution containing glucose, fructose, and trehalose, then three samples of haemolymph extract, and finally a second spot of the known solution. The chromatograms were developed for about 33 hr at 22°C in a descending solvent composed of ethyl acetate–acetic acid–formic acid–water: 18–3–1–4. The outer chromatograms of the known solutions and the centre chromatogram of the extract were treated by the silver nitrate method of TREVELYAN *et al.* (1950) to indicate the position of the spots. Sugars were eluted from the indicated areas of the remaining chromatograms and quantified.

RESULTS

The first series of analyses showed a considerable individual variability in blood carbohydrate levels and considerable heterogeneity between batches of larvae although these were progeny of many females taken at random from the colony. To reduce the effects of the latter one batch of larvae was distributed

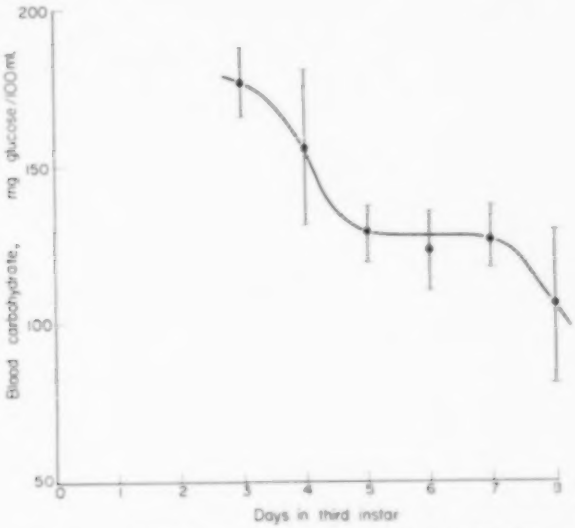

FIG. 1. Quantitative effect of age on blood carbohydrate in third-instar larvae.

over the various treatments under study. Generally six samples of twenty-four larvae, four per sample) were taken from each treatment and within these the standard deviations were about 20 per cent of the mean. The standard deviations of the individual blood samples must then have been about 40 per cent of the mean blood sugar level.

To seek an explanation for some of this variability the changes in carbohydrate concentration with age were determined. Analyses were started 3 days after the second larval moult and continued for 5 days until the larvae began to form puparia. The results are shown in Fig. 1. The carbohydrate level was relatively high on the third day (176 ± 10 mg/100 ml), decreased rapidly through the fourth day (155 ± 25 mg/100 ml) to the fifth (128 ± 8 mg/100 ml), then remained constant on the sixth and seventh (122 ± 12, and 127 ± 10 mg/100 ml respectively) before falling again on the eighth day (104 ± 24 mg/100 ml). Both of these changes have a high statistical significance ($P < 0.01$). Since it was customary to take blood on the fifth or sixth day of the stage, we could do no better than continue this practice in later experiments.

To determine the effects of dietary glucose levels on blood carbohydrates, the larvae were reared from birth on chemically defined diets containing different

amounts of glucose. The larvae grow well on glucose-free diets but added glucose increases the growth-rate to a maximum at about 0·5 per cent glucose; at 2·0 per cent glucose there is a definite retardation of growth (HOUSE and BARLOW, 1956). We therefore studied larvae reared on diets containing 0, 0·5, 1·0, and 2·0 per cent glucose. The experiment was repeated three times, six samples being obtained

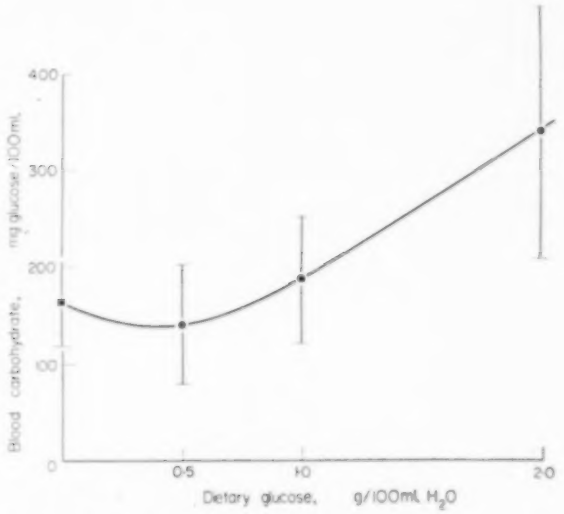

FIG. 2. Quantitative effect of dietary glucose concentration on blood carbohydrate in mature (6-day) third-instar larvae.

from insects on each of the four diets. Each time the blood carbohydrate was highest in the larvae growing on 2·0 per cent glucose, but in larvae on the other three diets the results were inconsistent. The results are combined and plotted in Fig. 2. There is no significant difference between the levels found on 0, 0·5, and 1·0 per cent glucose (161 ± 43, 140 ± 60, and 186 ± 66 mg/100 ml, respectively), but the blood carbohydrate level on 2·0 per cent glucose (337 ± 131 mg/100 ml) is higher than these by a statistically significant amount ($P < 0{\cdot}01$).

A similar experiment in which the blood samples were taken when the larvae were 3 days old in the third stage, and thus had an elevated blood carbohydrate level, showed that dietary glucose had a similar effect at this stage (see Fig. 3). There was, however, a statistically significant increase from insects reared on diets containing no sugar when 0·5 or 1·0 per cent glucose was fed as well as when 2·0 per cent glucose was fed and all the points appear to lie upon a straight line.

Finally, large samples of blood from larvae reared on diets containing 0, 0·5, and 2·0 per cent glucose were chromatographed so that the changes could be studied both qualitatively and quantitatively. In each experiment insects were

grown on each of the three diets, and samples of plasma were taken for total carbohydrate (10 per cent TCA filtrate), alcohol soluble carbohydrate (70 per cent alcohol filtrate), un-ionized carbohydrate, and chromatography. There was no difference between the amount of carbohydrate remaining in TCA and alcohol filtrates, and the amount lost during de-ionization (about 5 per cent) was the same

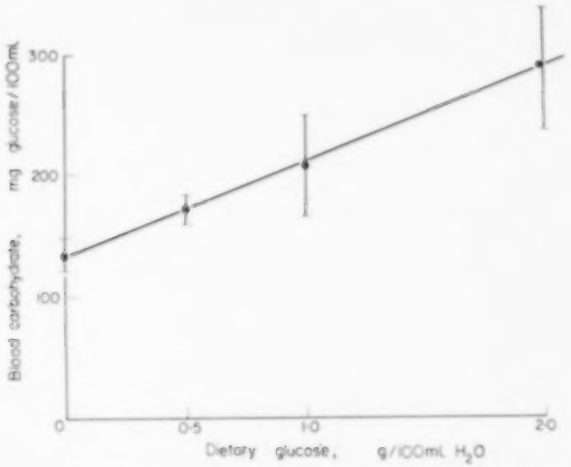

FIG. 3. Quantitative effect of dietary glucose concentration on blood carbohydrate in young (3-day) third-instar larvae.

regardless of the diet upon which the larvae were reared. This can be seen by expressing the alcohol soluble and un-ionized as a percentage of the total carbohydrate as follows (Table 1).

TABLE 1

Carbohydrate component	Glucose in the diet		
	0	0·5%	2·0%
Achohol soluble	102·0	101·7	100·4
Un-ionized	95·2	94·8	96·2

These results are averages from three repetitions of the experiment, and on the basis of this small number of samples there are no statistically significant differences.

When the chromatograms were treated by the silver nitrate method of TREVELYAN *et al.* (1950) the bulk of the reducing material appeared in the position of glucose. The identity of this sugar was supported by the formation of typical osazone crystals and its ready fermentability by brewer's yeast. Some material, probably a polysaccharide, remained at the origin. Although the extracts were

70 per cent alcohol this may have been unprecipitated glycogen. The next spot in descending order appeared in the disaccharide region of the chromatogram. This is presumed to be trehalose because it would not react with several carbohydrate indicators which react with common disaccharides. Its concentration in the blood is so low (*vide infra*) that no attempt has yet been made to isolate sufficient for complete characterization. The glucose spot was next, then a faint spot with the R_G of mannose, a darker spot with the R_G of fructose gave a positive ketose test, below this there were two more faint spots with mobilities in the pentose

Table 2

Glucose in diet	Recovered after chromatography	
	% of total carbohydrate	% of un-ionized carbohydrate
0	84·0	88·3
0·5	73·1	77·4
2·0	86·3	90·3

Table 3

Carbohydrates in blood	Glucose in diet		
	0	0·5%	2·0%
Glucose	87·7	84·3	86·3
Fructose*	5·4	5·7	7·1
Trehalose	1·5	3·3	1·0
Polysaccharide	3·7	5·2	3·1
Pentose	1·7	1·5	2·3

* These figures include a small amount of mannose.

region, the upper near arabinose, the lower near ribose. Since the original extraction with 70 per cent alcohol and the subsequent evaporation of solution on the chromatogram were accompanied by heating at an unknown pH some of the minor fractions may have been caused by partial breakdown of glucose. However, each of the spots was eluted and quantified with the exception of mannose and fructose which, because the spots were relatively large, were too close for complete separation and were eluted together. Four chromatograms were analysed from each blood sample. When these quantities were summed about 80 per cent of the total or 85 per cent of the un-ionized carbohydrate was accounted for. The experiment was repeated three times and the average recoveries were as shown in Table 2.

The low recovery on the 0·5 per cent diets verges on statistical significance at the 5 per cent level but there seems to be no logical reason for this, so to avoid prejudicing the quantities of the individual carbohydrates these are expressed in Table 3 as percentages of the recovered material.

Again, dietary glucose levels did not effect significant qualitative differences in the carbohydrates of the blood. In terms of absolute amounts 100 ml of blood of *Agria affinis* contains about 138 mg glucose, 9 mg fructose, 4 mg trehalose, 7 mg polysaccharide, and 3 mg pentose if the total blood carbohydrate is not elevated by a high glucose diet.

DISCUSSION

Buck (1953) discussed the wide variations that exist in the quantitative composition of insect bloods and the resultant impossibility of generalizing. Very few published results show individual variations within a species at specified stages of development. The blood of the adult female locust contains 24·1 ± 12·9 mg/100 ml glucose and 694·5 ± 99·3 mg/100 ml trehalose (Treherne, 1958a); these standard deviations are 54 per cent and 14 per cent, respectively, of the mean. The standard deviation is about 40 per cent of the mean in the present study. Although there are not enough data to calculate standard deviations for each of the component carbohydrates, glucose represents about 80 per cent of the total and thus must have approximately the same standard deviation. This is considerably greater than the standard deviation in mammals which, in man, amounts to 10 per cent of the mean for whole blood, 18 per cent for serum.

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Fig. 1 shows the quantitative changes in blood carbohydrate during the third larval stage. On the third day the carbohydrate level was relatively high and it decreased about midway through the stage (see Fig. 1, third to fifth day). These phenomena could be associated with chitin secretion. From the fifth to the seventh day the curve is flat, thus the variation discussed in the previous paragraph was not due to small differences in physiological age since the samples were taken on the sixth day. On the eighth day the carbohydrate concentration was further reduced. Cessation of feeding prior to pupation may be the cause as fasting is known to reduce blood sugar in insects. Usually at about this stage in the life cycle of insects there is an increase in reducing substances which Buck (1953) implies may be caused by an increase of phenolic substances rather than reducing carbohydrate. This has not been determined for *A. affinis*, but the above evidence supports Buck's implication.

Tsao and Richards (1952) showed that omission of glucose from the diet did not alter the ability of *Tribolium* and *Phormia* to form chitin. This is strong evidence that these insects can synthesize glucose but all sources of carbohydrate were not ruled out. House and Barlow (1956) showed that *A. affinis* can be grown on a glucose-free diet with only a slight retardation of growth. This diet is quite closely defined and the only apparent source of carbohydrate is the pentose in a small amount of ribonucleic acid (0·1 per cent). Fig. 2 shows an ability to synthesize glucose sufficient to maintain the carbohydrate level usual in mature third-stage larvae. However, compare Fig. 2, which shows the effect of dietary glucose on mature third-instar larvae (6 days), with Fig. 3, which shows the effect on young third-instar larvae (3 days). It appears that the latter are not able to

maintain their usual higher level of carbohydrate. This partial carbohydrate starvation may occur also at the earlier larval moult and may be responsible for the reduced rate of growth observed on glucose-free diets. Dietary levels of glucose higher than 1 per cent overcome the ability of the larvae to limit blood carbohydrate to the usual level. This is associated with a detrimental effect on growth of unknown mechanism (HOUSE and BARLOW, 1956). Some larvae were reared on diets containing up to 4 per cent glucose. On these diets the larvae grew poorly and erratically but a few samples of blood from a group reared on 3 per cent glucose (not included in Fig. 2) indicated that the blood glucose continues to rise and become more variable as the insects are subjected to greater stress.

Chromatographic analysis of blood of *A. affinis* larvae showed that about 80 per cent of the carbohydrate is glucose. In this way it differs markedly from the larvae of four lepidopterans and a hymenopteran studied by WYATT *et al.* (1956) and WYATT and KALF (1956, 1957), the adult locust studied by TREHERNE (1958a, b), and the glucose- or mannose-fed adults of *Phormia* studied by EVANS and DETHIER (1957) in all of which trehalose accounted for about 90 per cent of the blood carbohydrate. As might be expected, it most closely resembles the blood of larvae of *Phormia* which EVANS and DETHIER (1957) found to contain 100–160 mg per cent carbohydrate of which 70 per cent is glucose, 25 per cent polysaccharide, and little or no trehalose. It may differ from blood of *P. regina* larvae in having some fructose for, although EVANS and DETHIER reported on only a preliminary study, they did not detect fructose.

TREHERNE's (1958a) suggestion that diffusion of glucose across the gut wall is facilitated by its rapid conversion to trehalose could explain the observation of EVANS and DETHIER (1957) that trehalose increased sharply in the blood when sugars that could be used were fed. If this conversion takes place in the gut wall, it could explain the difference observed by EVANS and DETHIER between the effects of feeding or injecting the sugars. It could also conceivably occur in the larvae studied by WYATT and KALF (1957) which were similar in that trehalose is about 90 per cent of the blood carbohydrate. It is difficult, however, to suppose that this mechanism could function in the larvae of either *Phormia* or *A. affinis* which have high glucose and low trehalose concentrations in the blood. Indeed, our experiment indicates that feeding glucose causes a proportional increase in all carbohydrate components suggesting a state of dynamic equilibrium between them which maintains the proportionality.

Before speculating that this may represent a difference between larval and adult metabolism in sarcophagids such as *A. affinis* and *P. regina* it should be remembered that there is a fundamental difference between the experiments. The adult *P. regina* were starved, then given a single meal of sugar whereas the larvae of *A. affinis* were reared from birth on diets with various concentrations of glucose and so may have had time to adapt their carbohydrate metabolism to the level in the diet. Only a complete study of one of these insects in both adult and larval form can resolve the problem.

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UNTERSUCHUNGEN ÜBER DIE BEDEUTUNG DER CORPORA ALLATA FÜR DIE DIFFERENZIERUNG DER KASTEN BEI DER TERMITE *KALOTERMES FLAVICOLLIS* F.*

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Abstract—The role of the corpora allata in caste differentiation has been studied in the termite *Kalotermes flavicollis* by transplanting corpora allata of different donors into pseudergates in normal and in orphaned colonies.

When corpora allata from primary and secondary reproductives, from nymphs just before the alate moult, and from soldiers were implanted, a great number of the hosts differentiated into pre-soldiers. Transplants from pseudergates and last-stage nymphs which were not preparing for a moult, had no such effect, but those of pseudergates seemed to stimulate the production of supplementary reproductives.

The absence of the reproductives or of their pheromones had an accelerating effect upon pre-soldier differentiation.

In some cases intercastes between pre-soldiers and supplementary reproductives were produced. Their occurrence can be explained by an induction of a supplementary reproductive moult at a time when pre-soldier differentiation was already in progress. Intercastes between pre-soldiers and pseudergates have been produced experimentally by injecting ecdyson 10 days after the implantation of corpora allata from reproductives. Thus in soldier differentiation the moult is induced normally only some time after the onset of differentiation. Ligation experiments showed that the remarkable volume increase of the corpora allata before a supplementary reproductive moult has nothing to do with differentiation, but occurs in connexion with the production of gonadotropic hormone which in females stimulates growth of the oocytes.

All transplanted corpora allata with the exception of those of last-stage nymphs, had an inhibiting effect upon the pigmentation of eyes in supplementary reproductives. This typically juvenile effect was especially significant after transplanting corpora allata from pseudergates.

The corpora allata seem to produce two different hormones, which have different effects in caste differentiation. The juvenile hormone probably increases the competence for supplementary reproductive differentiation. The gonadotropic hormone on the other hand initiates pre-soldier differentiation.

EINLEITUNG

EINE normale Kolonie von *Kalotermes flavicollis* besteht zum grössten Teil aus ausgewachsenen Larven oder Pseudergaten (GRASSÉ und NOIROT, 1947; LÜSCHER, 1952a). Für die Pseudergaten bestehen bei jeder Häutung vier Möglichkeiten der Differenzierung: es können Ersatzgeschlechtstiere, Vorsoldaten, Nymphen

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oder wiederum Pseudergaten entstehen (LÜSCHER, 1952a). Für die Determination sind Ausseneinflüsse massgebend (Pheromone und Ernährungsfaktoren: KARLSON und LÜSCHER, 1959; LÜSCHER, 1960). Diese Ausseneinflüsse greifen bei den differenzierungsfähigen Pseudergaten in das endokrine System ein, welches letzten Endes die Art der Differenzierung bestimmt. Dabei spielen besonders die Corpora allata eine wichtige Rolle. Histologische Untersuchungen haben gezeigt, dass die Corpora allata kurz nach jeder Häutung ziemlich rasch anwachsen (LÜSCHER, 1957). Nach den Untersuchungen von SÄGESSER (1960) an *Leucophaea maderae* kann eine solche Volumenzunahme als vorübergehende Aktivierung gedeutet werden. Diese Aktivierung fällt zeitlich mit der Phase der höchsten Ersatzgeschlechtstier-Kompetenz (LÜSCHER, 1952b) zusammen und es stellt sich deshalb die Frage, ob die Ersatzgeschlechtstier-Kompetenz nicht von der Aktivität der Corpora allata abhängig ist. Auch nach der Auslösung einer Ersatzgeschlechtstierhäutung spielen die Corpora allata noch eine wichtige Rolle, da sie dann sehr rasch anwachsen und bei der Häutung 4–5 mal so gross sind wie bei einer Pseudergatenhäutung (LÜSCHER, 1957). In normalen, Geschlechtstiere enthaltenden Kolonien führt jedoch die Implantation aktiver Corpora allata in Pseudergaten nicht zur Entstehung von Ersatzgeschlechtstieren, sondern zur Differenzierung von Vorsoldaten (LÜSCHER, 1958). In Gegenwart der hemmenden Geschlechtstiere genügt also eine Aktivierung der Corpora allata allein nicht zur Auslösung der Ersatzgeschlechtstierhäutung. Es besteht aber noch die Möglichkeit, dass die Corpora allata die Kompetenz zur Ersatzgeschlechtstier-Differenzierung bei Abwesenheit der Geschlechtstiere beeinflussen. Um diese Frage zu prüfen, haben wir Corpora allata verschiedener Provenienz in Pseudergaten verwaister Kolonien implantiert, sodass gleichzeitig die relative Aktivität der Corpora allata verschiedener Entwicklungsstadien und Kasten geprüft werden konnte.

Auch die Frage nach der Bedeutung des raschen Anwachsens der Corpora allata nach Auslösung der Ersatzgeschlechtstierhäutung wurde untersucht. Da die Exstirpation der Corpora allata nicht gelungen ist, haben wir in verwaisten Kolonien bei Pseudergaten nach der Auslösung der Ersatzgeschlechtstierhäutung, jedoch noch vor dem Beginn der Volumenzunahme der Corpora allata, den Kopf abgeschnürt und dadurch Thorax und Abdomen dem Einfluss der Corpora allata entzogen.

MATERIAL UND METHODEN

Die für die Implantationsversuche in verwaisten Kolonien verwendeten Termiten wurden in Süditalien (Puglia) gesammelt. Die Schnürungsexperimente und die kombinierten Transplantations- und Injektionsversuche wurden an Termiten aus der Gegend von Banyuls sur mer (Pyr. or., Frankreich) durchgeführt. Es handelt sich hier um zwei Rassen, die sich morphologisch und physiologisch unterscheiden. Bei der italienischen Rasse tritt die Mutante mit schwarzem Pronotum der Imago (var. *fuscicollis* Becker) mehr oder weniger häufig auf, bei der französischen aber nie. Bei der italienischen Rasse erscheint die Pigmentierung der

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Augen im letzten Nymphenstadium früher und bei den Ersatzgeschlechtstieren ist sie viel häufiger und intensiver als bei den Termiten von Banyuls. Physiologische Unterschiede äussern sich beispielsweise in einer leichteren Ansprechbarkeit der italienischen Termiten auf den die Ersatzgeschlechtstierbildung stimulierenden Kopffaktor (LÜSCHER, 1956).

Die Versuchstermiten wurden individuell mit Farbmarken versehen und in flachen Beobachtungsnestern gehalten. Gefüttert wurde mit Agar-Buchensägemehl, dem in vielen Fällen zur besseren Sichtbarmachung der Darmentleerung vor der Häutung etwas Medizinalkohle (Merck) zugesetzt wurde. Manchmal haben wir auch nur Filtrierpapier verfüttert. Die Zuchttemperatur betrug durchwegs 26°C.

Die zu transplantierenden Organe wurden mit Nadeln in Periplaneta-Ringer (PANTIN) präpariert. In den meisten Fällen wurden die Corpora allata zusammen mit den Corpora cardiaca* und einem Teil des Nervus recurrens und manchmal mit kleinen Komplexen anderer Gewebe (Fettkörper, Bindegewebe, Teile des Dorsalgefässes) implantiert. Zur Injektion verwendeten wir eine modifizierte Apparatur nach EPHRUSSI und BEADLE. Die Kontrolltermiten wurden grösstenteils auch operiert (Injektion von Ringerlösung oder Implantation von Fett- oder Muskelgewebe). Durch Injektion von Ecdyson mit Hilfe einer Mikroinjektionseinrichtung konnten Häutungen ausgelöst werden (LÜSCHER und KARLSON, 1958). Die Ecdysonpräparate wurden uns freundlicherweise durch Herrn Dr. P. KARLSON (Physiologisch-Chemisches Institut der Universität München) zur Verfügung gestellt.

Alle Operationen wurden unter CO_2-Narkose durchgeführt.

Für die Operationen wurden nur weibliche Termiten verwendet und zwar Pseudergaten und Nymphen des ersten und zweiten Stadiums. Da die weiblichen Termiten bei der geschlechtlichen Differenzierung (Entstehung von Ersatzgeschlechtstieren und Interkasten) die Styli verlieren, konnte anhand dieses Kriteriums die Art der Differenzierung auch bei gestörter Pigmentierung der Augen festgestellt werden.

Die Kontrolltiere wurden in denselben Nestern gehalten wie die entsprechenden Versuchstermiten. In allen Fällen entstammten Versuchs- und Kontrolltiere der gleichen Stammkolonie. Um möglichst normale Verhältnisse in den Nestern zu verwirklichen, wurden auch immer einige männliche Larven oder Pseudergaten zugesetzt. Die geschnürten Termiten mussten einzeln gehalten werden, da sie sonst dem Kannibalismus zum Opfer gefallen wären. Da diese kein feuchtes Futter mehr aufnehmen konnten, wurden sie zur Verhinderung des Austrocknens in ein geschlossenes Gefäss mit einer relativen Luftfeuchtigkeit von 98 Prozent gebracht.

Zur Bestimmung des Volumens der Oocyten wurden mit Thionin gefärbte Totalpräparate der Ovarien hergestellt. Die Länge *l* und der mittlere Durchmesser *d* der 4 grössten Oocyten wurden gemessen. Der Einfachheit halber wurde das

* In früheren Versuchen hatten die implantierten Corpora cardiaca keinen Einfluss auf die Entstehung von Vorsoldaten.

Volumen nach der Formel $V = l \cdot \pi \cdot (d/2)^2$ berechnet. Der dadurch zustande kommende Fehler konnte vernachlässigt werden, da nur vergleichbare Werte notwendig waren.

DEFINITIONEN UND ABKÜRZUNGEN

Da gewisse Begriffe nicht immer im gleichen Sinne angewendet werden, halten wir es für nötig, diese so zu definieren, wie sie von uns aufgefasst werden. Ferner möchten wir zur Vereinfachung im Text einige Abkürzungen verwenden, die wir im Folgenden anführen:

C.a. = *Corpora allata*

L = *Larven*. Alle Entwicklungsstadien ohne Flügelanlagen werden als Larven bezeichnet. Da aber nach der 5.–7. Häutung das Wachstum aufhört, obschon in regelmässigen Abständen Häutungen durchgemacht werden (LÜSCHER, 1952a), und da diese Stadien nicht von denjenigen unterschieden werden können, welche durch regressive Häutungen aus Nymphen entstehen, ist es zweckmässig sie als

PE = *Pseudergaten* oder ausgewachsene Larven zu bezeichnen. Pseudergaten sind also nicht nur ausgewachsene Larven, sondern auch solche, die aus Nymphen durch Rückbildung (Regression) der Flügelanlagen entstanden sind.

N = *Nymphen* oder Larven mit Flügelanlagen. Sie entstehen aus Pseudergaten. Obschon es alle Übergänge in der Länge der Flügelanlagen gibt, ist es zweckmässig 2 Nymphenstadien zu unterscheiden (N_1 und N_2). Nur aus dem zweiten Nymphenstadium entstehen geflügelte Imagines. In kleinen Laboratoriumskolonien entstehen Nymphen recht selten. In der Natur ist ihre Entstehung jahreszeitlich bedingt.

IM = *Imago* oder geflügeltes Adulttier, das aus N_2 entsteht.

PG = *Primäres Geschlechtstier* (König oder Königin). Sie entstehen durch Flügelabwurf aus IM und gründen paarweise neue Kolonien.

EG = *Ersatzgeschlechtstiere* (reali di sostituzione; supplementary reproductives). Dies sind Pseudergaten oder Nymphen, die nach einer besonderen Häutung geschlechtsreif werden und König oder Königin in ihrer Funktion ersetzen. Sie unterscheiden sich von Pseudergaten und Nymphen äusserlich durch die starke Ausbildung der Sternalplatte des 7. Abdominalsegmentes und das Fehlen der Styli im weiblichen Geschlecht; durch die mehr oder weniger starke Pigmentierung der Augen und eine ziemlich variable Gelbfärbung der ganzen Cuticula bei beiden Geschlechtern. Die Prothorakaldrüsen degenerieren nach der EG-Häutung wie bei den Imagines. Sie können sich deshalb nicht mehr häuten.

VS = *Vorsoldaten* (soldati bianchi, white soldiers, or pre-soldiers). Dies sind Zwischenstadien zwischen Pseudergaten oder Nymphen und Soldaten. Ihr Kopf ist etwas in die Länge gestreckt, die Mandibeln sind weich, aufgebläht und turgeszent. Die linke Mandibel weist 3 Marginalzähne

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auf (diejenige der Larven, Pseudergaten, Nymphen, Imagines und Ersatzgeschlechtstiere hat nur 2 Marginalzähne). Der Vorsoldat nimmt nur ganz wenig Nahrung zu sich, die ihm von den Pseudergaten dargeboten wird. Er häutet sich nach 10–18 Tagen zum Soldaten weiter. Die äusseren Faktoren, welche zur Entstehung von Vorsoldaten führen sind nicht bekannt. In Laboratoriumskolonien ist die Vorsoldatenhäutung ein relativ seltenes Ereignis. In soldatenlosen Kolonien wurde 1 Vorsoldatenhäutung auf 50–200 andere Häutungen beobachtet.

S = *Soldaten.*

VS–L = *Interkaste*, Zwischenform zwischen Vorsoldat und Larve bezw. Pseudergat. Kopf und Mandibeln sind kürzer als beim normalen Vorsoldaten, doch weist die linke Mandibel 3 Marginalzähne auf.

VS–EG = *Interkaste*, Zwischenform zwischen Vorsoldat und Ersatzgeschlechtstier. Wie Vorsoldat-Larve, beim Weibchen jedoch sind die äusseren Geschlechtsmerkmale (7. Sternalplatte, Styli) adult. Die Augen sind manchmal pigmentiert. Kopf und Mandibeln können nahezu so lange sein wie beim Vorsoldaten.

Verwaiste Kolonie = Kolonie ohne Geschlechtstiere.

Kompetenz = Reaktionsbereitschaft der Termiten zur Differenzierung in bestimmter Richtung.

TRANSPLANTATIONSEXPERIMENTE IN VERWAISTEN KOLONIEN

C.a. verschiedener Spender wurden in 365 PE und N implantiert. Eine gleiche Zahl entsprechender Termiten dienten als Kontrollen. Alle Versuchstiere stammten aus etwa 40 Holzstücken, die in Süditalien gesammelt worden waren. Es wurden nur Termiten aus Kolonien verwendet, die ein Paar Geschlechtstiere enthielten. Die Operationen wurden von Mitte August bis Anfangs Dezember 1959 durchgeführt, und zwar für die verschiedenen Spenderklassen in sukzessiven Perioden.

Die Versuchskolonien wurden täglich kontrolliert, wobei tote und gehäutete Termiten registriert und dann entfernt wurden. In manchen Fällen sind die Versuchstiere an der Häutung gestorben. Der Charakter der Häutung konnte dann oft noch nach der Untersuchung der Mandibeln und der Genitalstyli erkannt werden. Diese Fälle figurieren in der Tabelle und in den Abbildungen unter den entsprechenden Häutungskategorien und nicht unter den gestorbenen Termiten.

Die Sterblichkeit war sowohl bei den Versuchs- als auch bei den Kontrolltermiten sehr hoch, besonders während der ersten 2–3 Tage als Folge der Operation. Aber auch später blieb die Sterblichkeit in beiden Gruppen höher als normal, wahrscheinlich infolge einer durch die Operation bedingten stärkeren Anfälligkeit gegenüber Krankheiten, durch die einzelne Kolonien oft in kurzer Zeit ganz verloren gingen. Dieser Sterblichkeit muss bei der Interpretation der Resultate Rechnung getragen werden, denn eine Häutung am Ende des Versuchs, wenn

nur noch wenige Tiere vorhanden sind, hat statistisch viel mehr Gewicht als eine solche am Anfang, wenn die Zahl der Individuen gross ist. Wir haben deshalb die täglichen Häutungsfrequenzen in %-Werten der zum betreffenden Zeitpunkt

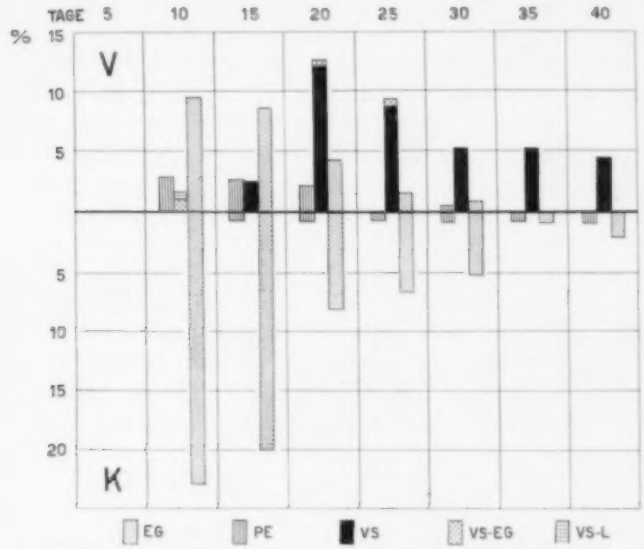

ABB. 1. Ergebnisse der Implantationsversuche in verwaisten Kolonien. Spender der Corpora allata: EG, IM und N_8 vor IM-Häutung. V = Versuch, K = Kontrolle. Die Säulen entsprechen den Prozentzahlen der in Intervallen von je 5 Tagen gehäuteten Termiten.

TABELLE 1—ZUSAMMENSTELLUNG DER ERGEBNISSE DER IMPLANTATIONSVERSUCHE IN VERWAISTEN KOLONIEN

Die Zahlen für die Kontrollen sind in Klammern angegeben

Spender des Implantats	Versuchsdauer Tage	Anzahl Termiten	Gestorben		Gehäutet						Nicht gehäutet
			1–4 Tg	Später	PE	EG	VS-L	VS-EG	VS	Total	
EG	40	159 (162)	37 (16)	44 (52)	14 (5)	15 (78)	1 (0)	4 (0)	34 (0)	68 (84)*	10 (10)
IM	40	53 (45)	4 (13)	8 (7)		24 (24)			14 (0)	38 (24)	3 (1)
PG	30	22 (26)	3 (3)	6 (4)	1 (1)	5 (11)			5 (0)	11 (12)	2 (7)
S	30	56 (56)	25 (17)	9 (13)	1 (0)	11 (17)			4 (0)	16 (17)	6 (9)
N_8 vor IM-Häutung	40	15 (19)	2 (1)	1 (5)		2 (7)			9 (0)	11 (7)	1 (6)
PE	26	48 (45)	13 (6)	15 (17)	1 (0)	17 (14)			1 (0)	19 (14)	1 (8)
N_8 nicht in Häutung	22	12 (12)	2 (1)	3 (5)		7 (6)				7 (6)	0 (0)
Total†		365 (344)	172 (149)		17 (6)	81 (151)	1 (0)	4 (0)	67 (0)	170 (158)*	23 (37)

* Inklusive 1 Imaginalhäutung.

† Das Total der Kontrollen entspricht nicht der Summe, da einige Termiten als Kontrollen für zwei verschiedenene, im gleichen Nest durchgeführte Versuche dienten.

theoretisch noch vorhandenen Termiten (Summe der gehäuteten und nicht gehäuteten Termiten) umgerechnet. Die Summe dieser Tagesfrequenzen ergab die Gesamtfrequenz. In Tabelle 1 sind die absoluten Werte zusammengestellt. In Abb. 1 und im Text beziehen wir uns jedoch—wenn nichts anders

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vermerkt ist—auf die korrigierten %-Werte. Die Resultate unserer Experimente sollen im Folgenden für jede Gruppe gesondert besprochen werden.

1. *Kontrollen.* Alle Kontrollen können zusammen betrachtet werden, da zwischen den einzelnen Gruppen keine signifikanten Unterschiede feststellbar sind. Es traten 151 EG-Häutungen und 6 PE-Häutungen auf. Eine N häutete sich zu einer IM. Es entstanden während der Beobachtungsperiode keine VS. Die ersten EG entstanden 6 Tage, die letzten 39 Tage nach Versuchsbeginn. Die höchste Frequenz wurde am 10. Tag erreicht. Unter Berücksichtigung der Sterblichkeit lässt sich eine EG-Frequenz für die 40 Tage dauernden Versuche von 64,7% errechnen. Dies ist ein relativ hoher Wert. LÜSCHER (1956) fand bei gleicher Versuchsdauer für nichtoperierte Termiten aus Banyuls eine EG-Frequenz von 49%, für Termiten aus Neapel nur 32%. Es ist deshalb nicht ausgeschlossen, dass der Operationsschock eine stimulierende Wirkung auf die EG-Differenzierung hat oder die EG-Kompetenz erhöht. Wenn die Zeit logarithmisch ausgedrückt wird, entspricht die Kurve der EG-Häutungsfrequenz approximativ einer Poisson-Verteilung. Die mittlere Dauer bis zur EG-Häutung beträgt 13,6 Tage. In keiner der 7 Gruppen von Kontrolltermiten weicht die Verteilung der EG-Häutungen und die mittlere Zeit zwischen Versuchsbeginn und EG-Häutung signifikant vom Durchschnitt ab.

2. *Ersatzgeschlechtstiere als Spender.* Zuerst haben wir C.a. aus EG transplantiert, deren Aktivität bereits nachgewiesen war (LÜSCHER, 1958). Meist wurden die EG unmittelbar oder wenige Tage nach der Häutung verwendet. Nur in einzelnen Fällen wurden auch ältere EG aus Kolonien, denen die Empfänger der Transplantate entnommen wurden, als Spender herangezogen.

Der Durchmesser der implantierten C.a. betrug $95{,}1 \pm 0{,}7\ \mu$, bei einem Minimum von $71{,}4\ \mu$ und einem Maximum von $129{,}3\ \mu$. Die Frequenzkurve entspricht einer Normalverteilung. Sie wurde anhand von 270 Messungen geprüft.

Die Operationen wurden von Mitte August bis Ende Oktober 1959 durchgeführt. Die Empfänger stammten aus über 30 Stammkolonien. Es ist wahrscheinlich, dass etwa gleich oft in allen Phasen des Häutungsintervalls operiert wurde, mit Ausnahme der letzten 7 Tage des Intervalls, in denen der Darm in Vorbereitung der Häutung schon entleert ist. Solche hell erscheinende PE und N wurden nicht verwendet.

Die zwischen dem 5. und 40. Versuchstag festgestellte Sterblichkeit betrug 36% bei den Versuchstermiten und 32% bei den Kontrollen. In beiden Gruppen machten je 10 Termiten während der Versuchsdauer von 40 Tagen keine Häutung durch. Wenn man die Mortalität berücksichtigt, häuteten sich 73,2% bei den Versuchstermiten und 68,3% bei den Kontrollen. In beiden Gruppen erfolgten also etwa gleich viel Häutungen. Die Art der Häutungen jedoch war verschieden: bei den Kontrollen entstanden 4,9% PE, 62,4% EG und 1,0% IM (1Tier); bei den Versuchstieren 14,6% PE, 12,3% EG, 41,2% VS und 5,1% Interkasten (1 VS–L und 4 VS–EG). Die Resultate dieses Versuchs sind zusammen mit denjenigen der unter 3 und 6 beschriebenen Versuche in Abb. 1 dargestellt.

Weder die Zeit des Auftretens noch die Verteilung der EG ist in den beiden Gruppen signifikant verschieden. Die VS jedoch traten viel später auf als die EG, im Mittel nach 24 Tagen. Auch nach dem 40. Tag wurden noch VS-Häutungen beobachtet, die letzte am 72. Tag nach der Operation. Die Verteilung des Auftretens der 34 VS-Häutungen entspricht angenähert einer Normalverteilung.

Auffallend ist, dass die Zahl der PE-Häutungen bei den Versuchstieren viel höher ist als bei den Kontrollen. Die meisten dieser PE-Häutungen traten viel früher auf als diejenigen bei den Kontrolltieren.

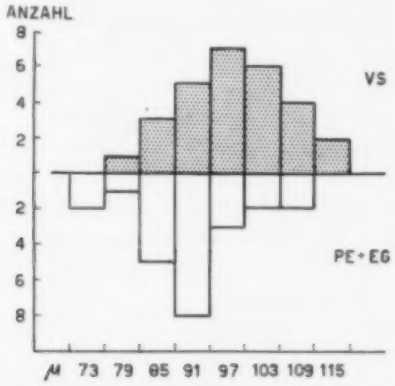

ABB. 2. Beziehung zwischen der Grösse der Implantate und der Art der resultierenden Häutung. Spender der Corpora allata : EG.

Die 5 Interkasten hatten alle VS-Mandibeln (linke Mandibel mit 3 Marginalzähnen; Abb. 5). Die zuerst, am 8. Tag aufgetretene, hatte stark reduzierte Mandibeln, besass aber Genitalstyli und ist somit als Zwischenform des Typs VS–L zu betrachten. Alle anderen besassen neben reduzierten Mandibeln auch keine Genitalstyli. Es handelte sich also um Zwischenformen zwischen VS und EG. Zwei davon häuteten sich am 9. Tag, je eine am 18. und 21. Tag. Die letztere starb während der Häutung, doch konnte das Fehlen der Styli und die Bezahnung der Mandibeln noch festgestellt werden. Keine der Interkasten hatte pigmentierte Augen.

Zwischen dem Geschlecht der Spender und dem Differenzierungsresultat besteht keine Beziehung; ebensowenig kann die Pigmentierung des Integumentes der Spender von Bedeutung sein: die transplantierten C.a. von 4 alten, stark pigmentierten EG aus den Stammkolonien ergaben 2 PE, 1 EG und 1 VS. Dagegen ist das Volumen der Implantate von Bedeutung. Die C.a. nach deren Implantation PE und EG entstanden, waren im Durchschnitt signifikant kleiner (92,8 μ resp. 92,2 μ Durchmesser) als diejenigen, welche VS erzeugten (Durchmesser 100,1 μ). Die C.a., deren Transplantation zur Differenzierung von Interkasten geführt hat, hatten durchschnittlich einen Durchmesser von 100,4 μ. Diese Resultate sind in Abb. 2 dargestellt. Sie bestätigen erneut, dass zwischen Volumen und Aktivität der C.a. eine Korrelation besteht.

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3. *Geflügelte Imagines als Spender.* Für diese Versuche wurden als Spender Geflügelte verwendet, die direkt aus dem Holz grösserer Kolonien entnommen wurden, die noch nicht geschwärmt hatten und die zum Teil noch unvollkommen pigmentiert waren. Einige wurden vor der Operation während einiger Tage isoliert aufbewahrt. Keiner der Spender hatte die Flügel abgeworfen.

Der Durchmesser der transplantierten C.a. betrug hier $89{,}7 \pm 1{,}0\ \mu$ mit einem Minimum von $65{,}6\ \mu$ und einem Maximum von $106{,}1\ \mu$ (52 Messungen).

Alle Versuche wurden während der ersten drei Wochen des Monats November 1959 durchgeführt. Die Empfängertermiten wurden 7–8 verschiedenen Stammkolonien entnommen. Die Versuchsdauer betrug 40 Tage.

Die Sterblichkeit zwischen dem 5. und 40. Tag betrug 16% bei den Versuchstieren, 22% bei den Kontrollen. Die Häutungsfrequenz betrug 77,3% bei den Versuchstermiten, 71,2% bei den Kontrollen. Während bei den letzteren ausschliesslich EG-Häutungen auftraten, entstanden bei den Versuchtermiten 45,0% EG und 32,3% VS.

In beiden Gruppen waren die mittlere Zeit bis zur EG-Häutung und die Verteilung der EG-Häutungen gleich. Die VS häuteten sich durchschnittlich nach 24,3 Tagen; der letzte am 36. Tag. Gegenüber den Versuchen mit EG-Spendern zeigten sich hierin keine Unterschiede.

4. *Primäre Geschlechtstiere als Spender.* Die verwendeten PG entstammten kleinen, einige Monate vor Versuchsbeginn gegründeten Kolonien, die etwa 10 L und in einigen Fällen einen S enthielten. Zur Zeit der Entnahme der PG waren in den Nestern keine Eier vorhanden.

Der mittlere Durchmesser der transplantierten C.a. betrug $96{,}4 \pm 1{,}8\ \mu$ mit einem Minimum von $73{,}3\ \mu$ und einem Maximum von $127{,}4\ \mu$ (28 Messungen). Die Empfänger entstammten 4 oder 5 grossen Kolonien. Die Versuche wurden im November 1959 durchgeführt und dauerten 30 Tage.

Von 13 überlebenden Versuchstermiten häuteten sich 5 EG, 5 VS und 1 PE; von 19 Kontolltermiten 11 EG und 1 PE. Trotz der kleinen Zahl von Versuchen ist der Unterschied gesichert.

5. *Soldaten als Spender.* Es wurden ältere, ziemlich grosse Soldaten mit dunkel gefärbten Köpfen verwendet, die alle den Stammkolonien entnommen wurden.

Der Durchmesser der transplantierten C.a. betrug $76{,}4 \pm 0{,}9\ \mu$ mit einem Minimum von $59{,}8\ \mu$ und einem Maximum $94{,}6\ \mu$ (73 Messungen).

Die Operationen wurden Ende November durchgeführt und die Empfänger wurden aus mehreren Stammkolonien entnommen. Die Versuchsdauer betrug 30 Tage.

Von 22 überlebenden Versuchstermiten häuteten sich 11 EG, 4 VS und 1 PE; von 26 Kontrolltermiten 17 EG.

6. *Nymphen vor der Imaginalhäutung als Spender.* Die für diese Experimente verwendeten N_2 hatten grosse, schwarze Augen und aufgeblähte, abstehende Flügelanlagen. Ferner hatten sie als Anzeichen der herannahenden Häutung den Darm entleert. Es handelte sich also um Tiere, die wenige Tage später eine Imaginalhäutung durchgemacht hätten.

Der Durchmesser der implantierten C.a. betrug 80,4 ± 3,1 μ bei einem Minimum von 67,5 μ und einem Maximum von 92,6 μ (9 Messungen).

Die Operationen wurden zu verschiedenen Zeiten von August bis Oktober 1959 durchgeführt. Die Versuchsdauer betrug 40 Tage.

Von 12 überlebenden Versuchstieren häuteten sich 2 EG und 9 VS; von 13 Kontrolltermiten 7 EG. Die Resultate bei Versuchs- und Kontrolltermiten sind signifikant verschieden.

7. *Pseudergaten vor der Häutung als Spender.* Für dieses Experiment verwendeten wir PE und N_1, die den Darm entleert hatten, also kurz vor der Häutung standen. Sie hatten keine pigmentierten Augen und wurden aus Kolonien entnommen, die ein Paar funktionelle Geschlechtstiere enthielten.

Die Grösse der transplantierten C.a. wurde nicht gemessen. Sie waren kleiner als alle für die bisher geschilderten Versuche verwendeten Drüsen. Wir haben deshalb in einigen Fällen 2 Paar C.a. in ein Individuum implantiert.

Die Operationen wurden Ende November und Anfangs Dezember durchgeführt. Die Empfänger entstammten 2 Stammkolonien. Die Versuchsdauer betrug 26 Tage.

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Von 20 überlebenden Versuchstermiten häuteten sich 17 EG, 1 PE und 1 VS; von 22 Kontrolltermiten 14 EG. Die Transplantate vermochten also keine VS-Differenzierung zu induzieren, doch scheinen sie in diesen Versuchen die EG-Frequenz erhöht zu haben (P 0,05). Eine einzige VS-Häutung kann nicht von Bedeutung sein, da auch in normalen Kolonien gelegentlich VS entstehen.

8. *Nymphen des 2. Stadiums, die sich nicht in Häutung befanden, als Spender.* Diese wenigen Versuche wurden hauptsächlich als Kontrollen durchgeführt. Es wurden N_2 als Spender verwendet, die keinerlei Anzeichen einer bevorstehenden Häutung zeigten. Da sich am 22. Tag alle überlebenden Versuchs- und Kontrolltiere zu EG gehäutet hatten, wurde der Versuch abgebrochen. Ein Unterschied zwischen den Gruppen konnte nicht nachgewiesen werden.

Der Einfluss der Transplantate auf die Differenzierung der Kasten in den verwaisten Kolonien ist evident. (Zusammenstellung der Resultate in Tabelle 1 und in Abb. 1 und 3.) Die implantierten C.a. aus EG, PG, IM, S, und N_2 vor der Imaginalhäutung haben alle eine Herabsetzung der EG-Frequenz und die Differenzierung von VS zur Folge, wobei die Zahl der Häutungen praktisch konstant bleibt. Dies bedeutet, dass die meisten VS aus PE und N entstanden sind, die sich ohne Implantat zu EG differenziert hätten. Von allen diesen Spendern hatten die C.a. der S die geringste Wirkung.

Würde der Zeitpunkt der Häutung durch das Implantat und durch die Verwaisung der Kolonie nicht beeinflusst, so müsste man annehmen, dass sowohl die Frequenz der PE- Häutungen als auch die Frequenz der VS-Häutungen während der ganzen Versuchsdauer konstant bleiben sollte, wie dies für die PE-Frequenz in den Kontrollversuchen mit Geschlechtstiere enthaltenden Kolonien der Fall war (vergl. Abb. 4). In den Versuchen, die am meisten VS ergaben, deren Resultate in Abb. 1 dargestellt sind, sind jedoch in der ersten

Periode ziemlich viele PE entstanden, sehr viel mehr als bei den Kontrollen. Es ist also wahrscheinlich, dass bei diesen Termiten die Häutung infolge der Verwaisung zwar ausgelöst wurde, dass aber die implantierten C.a. die EG-Differenzierung verhindert haben. Für die entstandenen VS ist eine Anhäufung um den 20. Tag festzustellen. Wie wir noch sehen werden, treten in Geschlechtstiere enthaltenden

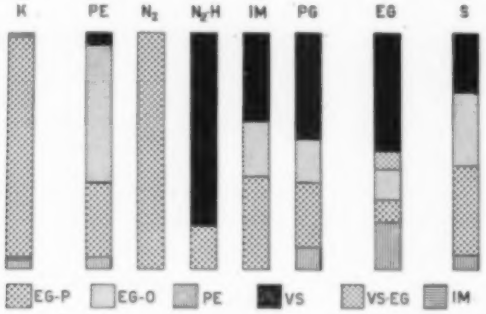

ABB. 3. Ergebnis der Häutungen nach Transplantation von Corpora allata verschiedener Spender. Über den Säulen ist der Spender angegeben. K = Kontrolle; N_2 = Nymphen des 2. Stadiums die sich nicht in Häutung befanden; N_2-H = Nymphen vor IM-Häutung; EG-P = EG mit pigmentierten Augen; EG-O = EG ohne pigmentierte Augen.

Kolonien die meisten VS erst um den 40. Tag auf. Die Entfernung der Geschlechtstiere hat demnach eine beschleunigende Wirkung auf die Differenzierung der VS. Es ist nicht ausgeschlossen, dass durch die Verwaisung allgemein die Häutungstendenz erhöht wird.

Eine andere Wirkung hatten die C.a. aus PE vor der Häutung. Sie scheinen die EG-Produktion zu stimulieren, also wahrscheinlich die EG-Kompetenz zu erhöhen. Dieses Ergebnis ist schwach gesichert und bedarf noch einer weiteren Nachprüfung. Keinerlei Wirkung wurde bei den C.a. aus gewöhnlichen N_2 beobachtet. Es muss allerdings betont werden, dass eine eventuelle Stimulierung der EG-Differenzierung aus dem Resultat nicht ersichtlich wäre, da sich ja auch alle Kontrolltermiten zu EG häuteten.

Bei den unter den Versuchstermiten entstandenen EG zeigte sich eine deutliche Wirkung der Implantate auf die Pigmentierung der Augen. Bei der italienischen Rasse von *Kalotermes flavicollis* erscheint die Augen-Pigmentierung meist 2–3 Tage vor der EG-Häutung, selten erst am Vortag der Häutung. Unter den 151 beobachteten EG aus den Kontrollgruppen hatten nur zwei unpigmentierte Augen. Das Unterbleiben der Pigmentierung ist also unter normalen Bedingungen ein seltenes Ereignis, das vielleicht auf eine hormonale Störung zurückzuführen ist.

Unter den nach Implantation von C.a. entstandenen EG war das Fehlen der Augen-Pigmentierung ziemlich oft zu beobachten. Es zeigte sich auch eine bemerkenswerte Variation in der Intensität der Färbung: vom intensiven braunschwarz über braun, hellbraun, gelblich bis zum vollkommenen Fehlen jeder Pigmentierung. In unserer Zusammenstellung (Tabelle 2, Abb. 3) wurden die

Individuen wegen der Schwierigkeit der Klassierung in Gruppen mit verschieden intensiver Pigmentierung nur in zwei Kategorien aufgeteilt. Im Ganzen gehören etwas weniger als die Hälfte der Versuchstiere zur Kategorie 'nicht pigmentiert'. Bei Betrachtung der einzelnen Versuchsgruppen ergeben sich bemerkenswerte Unterschiede. Die höchsten Frequenzen der 'nicht pigmentierten' traten auf, wenn

TABELLE 2—PIGMENTIERUNG DER AUGEN BEI ERSATZGESCHLECHTSTIEREN NACH IMPLANTATION VON CORPORA ALLATA

Spender des Implantats	Ersatzgeschlechtstiere	
	Total	ohne pigmentierte Augen
EG	15	9 = 60 %
IM	24	9 = 37,5%
PG	5	2 = 40 %
S	11	5 = 45,5%
PE	17	11 = 65 %
N_2	7	0
N_2 vor IM-Häutung	2	0
Kontrollen	151	2 = 1,3 %

EG und PE als Spender verwendet wurden; etwas weniger hohe bei IM, PG und S als Spender. C.a. aus N_2 waren wirkungslos in beiden Versuchsgruppen, wobei allerdings die erste Gruppe zu klein ist, um Bedeutung zu haben.

TRANSPLANTATIONSEXPERIMENTE IN GESCHLECHTSTIERE ENTHALTENDEN KOLONIEN

Zum Vergleich haben wir die Resultate früherer Versuche an Termiten aus Banyuls, die z.T. schon publiziert sind, in der gleichen Art ausgewertet und in Abb. 4 dargestellt. Es handelt sich um 63 Versuchstermiten, PE und N_2, die aus 6 Stammkolonien entnommen wurden und in die je ein Paar C.a. aus frisch gehäuteten EG implantiert wurde. Sie wurden in 6 Versuchsnestern zusammen mit insgesamt 158 entsprechenden Kontrolltermiten gehalten. Letztere wurden nicht operiert. Die Versuchsdauer betrug 50 Tage.

Die Sterblichkeit war bei den Versuchstermiten hoch, nämlich 46% gegenüber 9% bei den Kontrollen. Wir haben deshalb wie bei den schon beschriebenen Versuchen bei der Berechnung der Häutungsfrequenzen die Mortalität berücksichtigt und uns immer auf die zu jedem Zeitpunkt noch vorhandenen gehäuteten und nicht gehäuteten Termiten bezogen. Die Gesamtfrequenz wurde durch Addition der Einzelfrequenzen errechnet.

In 3 von 6 Versuchskolonien traten bei Versuchs- und Kontrolltermiten EG auf. Dies sollte eigentlich in Geschlechtstiere enthaltenden Kolonien nicht vorkommen. Es ist wahrscheinlich auf eine vorübergehende Störung der Hemmwirkung der Geschlechtstiere zurückzuführen. Es traten auch nur in den ersten 18 Tagen nach Versuchsbeginn EG auf. Später haben also alle Geschlechtstiere

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normal funktioniert. Die EG-Frequenzen waren bei Versuchs- und Kontrolltermiten etwa gleich hoch (Versuch 8,9%, Kontrolle 9,6%).

Bei den Versuchstermiten wurden 25, bei den Kontrolltermiten 63 Häutungen beobachtet. Die Häutungsfrequenz ist bei den Versuchen mit 65% bedeutend höher als bei den Kontrollen mit 42%.

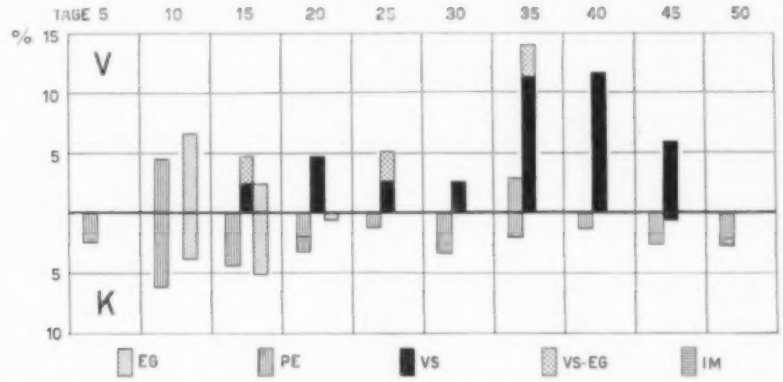

ABB. 4. Ergebnisse der Implantationsversuche in Geschlechtstiere enthaltenden Kolonien. Spender der Corpora allata: EG. Weitere Erklärungen siehe unter Abb. 1.

Die Frequenz der PE- und IM-Häutungen ist bei den Kontrolltermiten während der ganzen Versuchsdauer ungefähr gleich. Bei den Versuchstermiten ist sie zu Beginn des Versuchs etwa gleich hoch wie bei den Kontrollen. Nach dem 10. Tag wurde jedoch nur noch eine PE-Häutung beobachtet. Dafür traten bei den Versuchstermiten vom 14. Tag an VS, VS-EG und VS-L-Häutungen auf, und zwar bis zum 30. Tag etwa in gleicher Frequenz wie PE-Häutungen bei den Kontrollen. Zwischen dem 31. und dem 40. Tag jedoch steigt die VS-Frequenz sehr stark an, um dann wieder abzusinken. Diese erhöhte VS-Frequenz ist für die Erhöhung der allgemeinen Häutungsfrequenz bei den Versuchstermiten verantwortlich.

Der Versuch lässt sich folgendermassen interpretieren. Die implantierten C.a. haben keinen Einfluss auf die Termiten, deren Larvenhäutung kurz nach Versuchsbeginn induziert wird. Sie haben auch keinen Einfluss auf die EG-Differenzierung, die in Termiten mit sehr hoher Kompetenz zu Beginn des Versuchs ausgelöst wird. Bei den übrigen wird eine VS-Differenzierung induziert. Dabei machen diejenigen, welche schon nahe am Ende des Häutungsintervalls stehen, die Häutung zum normalen Zeitpunkt durch (10.–30. Tag gleiche Frequenz für VS bei Versuch wie für PE bei Kontrolle). Bei den übrigen Termiten wird durch das Implantat nicht nur die VS-Differenzierung ausgelöst, sondern auch der Zeitpunkt der Häutung vorverlegt, sodass die Häutung eintreten kann, wenn die VS-Differenzierung abgeschlossen ist. Hierfür spricht die Anhäufung von VS-Häutungen zwischen dem 30. und 40. Tag.

DIE INTERKASTEN UND DIE EXPERIMENTELLE AUSLÖSUNG IHRER ENTSTEHUNG

Nach Implantation von C.a. aus EG sind in verwaisten Kolonien 5 Interkasten entstanden, 1 VS-L und 4 VS-EG. Auffallenderweise entstanden 3 dieser Interkasten früher als die ersten VS. Dies deutet darauf hin, dass bei diesen Termiten die Häutung ausgelöst wurde, nachdem die VS-Differenzierung schon begonnen hatte, dass jedoch diese aufgezwungene Häutung eintrat, bevor die Differenzierung abgeschlossen war. Auch bei den z.T. schon publizierten

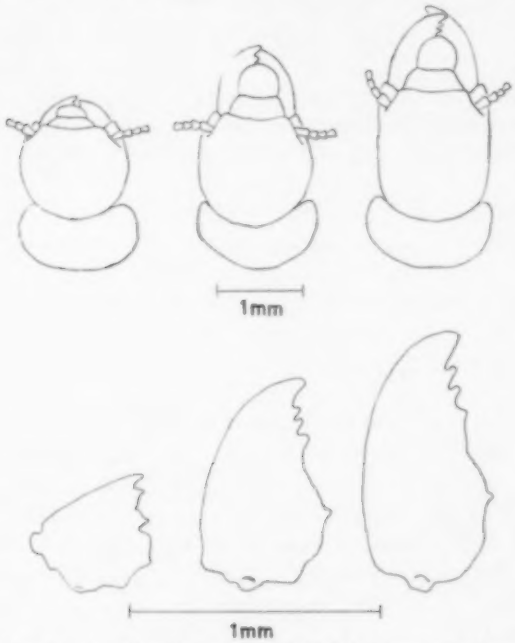

ABB. 5. Kopf und linke Mandibel eines PE (links), einer experimentell erzeugten Interkaste (VS-L) und eines normalen VS (rechts).

Versuchen (LÜSCHER, 1958) in normalen, Geschlechtstiere enthaltenden Kolonien von Termiten aus Banyuls traten 3 Interkasten auf, und zwar auch hier ein VS-EG früher als alle VS.

Um die Frage zu prüfen, ob die Auslösung einer Häutung nach begonnener Differenzierung zur Ausbildung von Interkasten führen kann, haben wir in 14 PE-Weibchen (Banyuls) C.a. aus frisch gehäuteten EG implantiert und nach 10 Tagen 5 oder 25 Calliphora-Einheiten Ecdyson in 1 μl Ringer injiziert. Bei 10 der Versuchstiere trat die Häutung 7–14 Tage nach der Injektion ein: es entstanden 2 EG, 2 PE und 6 VS-L. Von diesen 6 Interkasten konnte sich allerdings nur eine selbständig häuten. Diese ist in Abb. 5 dargestellt. Bei den übrigen wurde die schon abgehobene alte Cuticula entfernt. Bei allen konnten 3 Marginalzähne an der linken Mandibel und Styli am Abdomen festgestellt werden.

Da bei sofortiger Injektion von Ecdyson oder bei Injektion 4–5 Tage nach der Transplantation der C.a. weder VS noch Interkasten entstanden sind (mit Ausnahme eines VS-EG, das sich jedoch erst 22 Tage nach der Injektion häutete und dessen Häutung somit nicht durch das Ecdyson ausgelöst worden sein kann; LÜSCHER, 1958), muss man annehmen, dass nach der Implantation der C.a. eine gewisse Zeit (5 Tage) benötigt wird, um die Determination der VS-Differenzierung auszulösen, dass die Differenzierung selbst normalerweise vor der Auslösung der Häutung einsetzt und dass die Häutung erst ausgelöst wird, wenn der Abschluss der VS-Differenzierung vor der Häutung gewährleistet ist.

Das Auftreten von VS-EG in unseren Versuchen kann demnach so gedeutet werden, dass in diesen Fällen die VS-Differenzierung schon eingesetzt hatte, als eine EG-Differenzierung induziert wurde, bei der ja immer eine Häutung ausgelöst wird. Da aber das Auftreten dieser VS–EG auch in verwaisten Kolonien relativ selten war, ist anzunehmen, dass mit der Determination der VS-Differenzierung die EG-Kompetenz sehr stark herabgesetzt wird.

Die bei *Zootermopsis* beschriebenen fertilen Soldaten, die sog. 'reproductive soldiers' (LIGHT, 1942/43) scheinen ebenfalls VS–EG zu sein, die sich nicht mehr häuten. Sie entstehen in verwaisten Kolonien und sind wohl auch durch die Auslösung einer EG-Häutung nach begonnener VS-Differenzierung zu erklären. Der von HEATH (1927) abgebildete 'fertile soldier' scheint allerdings eine Zwischenform zwischen S und EG zu sein.

SCHNÜRUNGSEXPERIMENTE

Zur Untersuchung des Einflusses der C.a. nach Auslösung einer EG-Häutung wurden in zwei verwaisten Kolonien aus Banyuls in den ersten Tagen nach Ansetzen die weiblichen PE nach der Darmentleerung geschnürt. Diese Operation ist möglich, da nach der Darmentleerung keine Nahrung mehr aufgenommen wird. Die Kontrollen wurden alle 24 Stunden durchgeführt; der Zeitpunkt der Schnürung liegt daher zwischen 0 und 24 Stunden nach der Darmentleerung. Erfahrungsgemäss häuten sich nahezu alle PE, die in den ersten 6 Tagen nach Entfernung der Geschlechtstiere den Darm entleeren, zu EG. Die Ligatur wurde immer zwischen Kopf und Prothorax angelegt und fest angezogen. Der Kopf starb in allen Fällen am ersten Tag nach der Operation ab und wurde dann meist entfernt. Da nach histologischen Untersuchungen (LÜSCHER, 1957) das starke Wachstum der C.a. erst einen Tag nach der Darmentleerung einsetzt, darf als sicher gelten, dass es in unseren Versuchen nicht schon vor der Schnürung begonnen hatte.

Von 16 geschnürten weiblichen PE starben in den ersten fünf Tagen 5 Individuen. Von den übrigen waren am 6. Tag 8 und am 7. Tag 3 häutungsbereit. Die Häutungsbereitschaft liess sich daran erkennen, dass die Häutungsflüssigkeit resorbiert und der Zwischenraum zwischen der alten und der neuen Cuticula teilweise mit Luft gefüllt war. Da die kopflosen Termiten die Haut nicht selbst sprengen konnten, wurde die alte Cuticula abgelöst. Alle so gehäuteten Termiten waren noch in gutem Zustand und reagierten auch nach der Häutung mit koordinierten Gehbewegungen auf mechanische Reizung. Sie wurden teils sofort, teils

2 Tage nach der Häutung fixiert. Zu diesem Zeitpunkt hatte sich ihr Zustand, wahrscheinlich infolge des bei der Häutung eingetretenen Wasserverlustes, stark verschlechtert.

Sämtliche 11 gehäuteten Termiten hatten grosse Sternalplatten und bei allen fehlten die Styli. Sie hatten sich also äusserlich zu normalen EG differenziert. Auffallend war ferner eine aussergewöhnlich starke braun-gelbe Pigmentierung

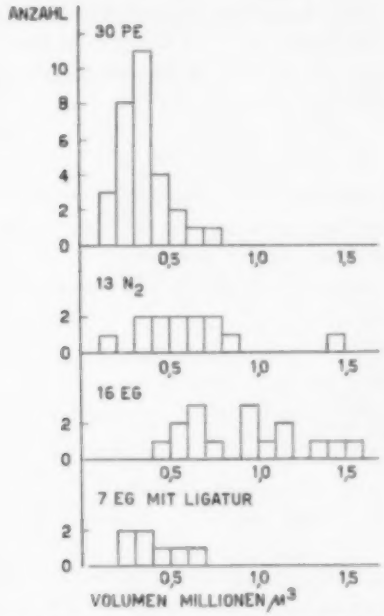

ABB. 6. Das Volumen der 4 grössten Oocyten bei 30 PE, 13 N_2, 16 EG und bei 7 geschnürten EG.

des ganzen Integumentes bei 5 Tieren. Eine solche Pigmentierung tritt normalerweise bei Ersatzgeschlechtstieren erst Wochen bis Monate nach ihrer Entstehung ein.

Als Kontrollen wurden 9 weibliche PE und N_2 aus normalen, Geschlechtstiere enthaltenden Kolonien nach der Darmentleerung geschnürt. Bei 6 Überlebenden waren nach Entfernung der alten Cuticula Styli vorhanden, und die dunkelgelbe Pigmentierung des Integuments trat nicht ein. Bemerkenswert ist, dass trotz der Abschnürung der C.a. in keinem Falle eine Differenzierung in imaginaler Richtung feststellbar war. Bei den N_2 trat sogar eine Regression der Flügelanlagen ein. Anscheinend sind die C.a. 1–2 Tage nach der Auslösung der Häutung für die larvale Differenzierung nicht mehr notwendig. Diese wird wohl schon vor der Auslösung der Häutung festgelegt.

Für die EG-Differenzierung lässt sich aus diesem Versuch schliessen, dass die Aktivierung der C.a. nach der Auslösung der Häutung keinen Einfluss auf die

äussere Differenzierung hat. Einzig eine Hemmung der Pigmentierung des Integumentes dürfte den C.a. zugeschrieben werden. Die Bedeutung der Aktivierung der C.a. liegt jedoch in einer Wirkung auf die Eireifung in den Ovarien. Wir haben die Ovarien von 7 Versuchstieren untersucht und für jedes das Volumen der 4 grössten Oocyten bestimmt. Es entspricht mit $0{,}40 \pm 0{,}056$ Millionen μ^3 demjenigen von frischgehäuteten PE mit $0{,}36 \pm 0{,}082$ Millionen μ^3, während die 4 grössten Oocyten bei frisch gehäuteten EG auf $0{,}92 \pm 0{,}084$ Millionen μ^3, d.h. auf mehr als das doppelte Volumen angewachsen sind (vergl. Abb. 6). Die C.a. haben also bei ihrem plötzlichen Anwachsen nach der Auslösung der EG-Häutung eine gonadotrope Wirkung und stimulieren das Wachstum der Oocyten in den Ovarien.

DISKUSSION

Unsere Versuche haben gezeigt, dass durch Implantation von C.a. in PE sowohl in Geschlechtstiere enthaltenden als auch in verwaisten Kolonien in einem hohen Prozentsatz VS erzeugt werden können.

Für die auf die Implantation folgende Differenzierung sind mindestens drei Faktoren von Bedeutung:

(a) der physiologische Zustand des Empfängerindividuums
(b) der physiologische Zustand der Kolonie und
(c) der physiologische Zustand der implantierten C.a.

(a) Der physiologische Zustand des Empfängerindividuums ist wahrscheinlich zur Hauptsache abhängig vom Alter innerhalb des Häutungsintervalls. Die Kompetenz zur EG-Differenzierung ist zu Beginn des Intervalls sehr hoch und sinkt dann zuerst rasch, später langsam gegen Null (LÜSCHER, 1952b). Es ist wahrscheinlich, dass die Kompetenz bei jedem Individuum allmählich abnimmt und erst nach der Determination der nächsten PE-Häutung erlischt. Dafür spricht die sehr hohe EG-Frequenz in unseren Versuchen mit verwaisten Kolonien, bei denen die neu entstandenen EG immer entfernt wurden. Werden, wie dies in den früheren Versuchen geschehen ist, die neu entstandenen EG in der Kolonie belassen, so ist die EG-Frequenz bedeutend niedriger. Es ist also anzunehmen, dass bei hoher EG-Kompetenz eine nur wenige Tage dauernde Verwaisung zur EG-Determination genügt, und dass bei abnehmender Kompetenz zunehmende Verwaisungszeiten für eine Determination notwendig sind. Auch die VS-Kompetenz dürfte sich ähnlich verhalten. Da die VS-Frequenz in den verwaisten Kolonien zusammen mit der EG-Frequenz etwa der bei den Kontrollen aufgetretenen EG-Frequenz entspricht, dürfte die VS-Kompetenz etwa der maximalen EG-Kompetenz bei langdauernder Verwaisung entsprechen.

(b) Der physiologische Zustand der Kolonie kann ganz allgemein auf die EG-Kompetenz einwirken. Es konnte z.B. nachgewiesen werden, dass die EG-Kompetenz bei günstiger Ernährungslage in individuenreichen Kolonien erhöht wird. In ähnlicher Weise dürfte auch die VS-Kompetenz von der Ernährungslage der Kolonie abhängig sein. Zur Hauptsache jedoch wirkt sich das

Fehlen oder Vorhandensein von Geschlechtstieren auf den physiologischen Zustand der Kolonie aus. Durch das Fehlen der Geschlechtstiere oder ihrer Pheromone (Ektohormone) wird die EG- Differenzierung bei kompetenten PE und N ausgelöst. Der Einfluss der Verwaisung auf die VS-Differenzierung nach C.a.-Implantation zeigt sich deutlich beim Vergleich der Versuche mit und ohne Geschlechtstiere (Abb. 1 und 4). In den verwaisten Kolonien sind die VS viel früher entstanden. Das Fehlen der Geschlechtstiere hat also eine beschleunigende Wirkung auf die VS-Differenzierung, vielleicht durch eine Erhöhung der Häutungsbereitschaft. Natürlich ist der physiologische Zustand der Empfängertermite von demjenigen der ganzen Kolonie abhängig, doch reagiert sie je nach ihrem individuellen Zustand verschieden.

TABELLE 3—ZUSAMMENSTELLUNG DER HORMONWIRKUNGEN DER IMPLANTIERTEN CORPORA ALLATA

Spender der C.a.	Erwartete Hormonart		Wirkung		
			Differenzierung von		Hemmung der Pigmentierung der Augen
	Juvenil	Gonadotrop	VS	EG	
PE	+	0	0	(+)	+ +
N_2	0	0	0	0	0
N_2 vor IM-Häutung	0	?	+ +	0	0
IM	0	+	+	0	+
PG	0	+	+	0?	+
EG	0	+	+ +	0	+
S	?	?	+	0?	+

(c) Der Einfluss des physiologischen Zustands der C.a. geht deutlich aus der Zusammenstellung über die Grösse der Implantate hervor (Abb. 2). Grössere Implantate bewirken zumeist VS-Differenzierung. Kleinere C.a. vermochten die EG-Differenzierung nicht zu verhindern. Ferner weisen die unterschiedlichen Resultate bei Transplantationen von C.a. verschiedener Entwicklungsstadien und Kasten auf die Bedeutung des physiologischen Zustands der implantierten C.a. hin (Abb. 3).

Wenn wir annehmen, dass die C.a. mindestens zwei verschiedene Hormone, ein Juvenilhormon und ein gonadotropes Hormon produzieren (die Versuche von SÄGESSER and *Leucophaea* sprechen gegen die Identität dieser Hormone), so produzieren die als Spender verwendeten PE Juvenilhormon, die N_2 kein Hormon, die IM, PG und EG aber gonadotropes Hormon. Für die EG geht dies aus den Schnürungsversuchen hervor, da das Wachstum der Oocyten nach Abschnürung des Kopfes mit den C.a. unterbleibt. Die C.a. der N_2 vor der IM-Häutung produzieren sicher kein Juvenilhormon, eventuell aber schon gonadotropes Hormon. Bei den C.a. der S lässt sich die Art der Produktion nicht voraussagen.

Wie die Zusammenstellung in Tabelle 3 zeigt, hatten die C.a. von denen eine Juvenilhormonwirkung erwartet werden durfte (PE) keine Wirkung auf die

Differenzierung von VS, eine stark hemmende Wirkung auf die Pigmentierung der Augen bei den entstandenen EG und möglicherweise eine stimulierende Wirkung auf die Differenzierung von EG. Da die Pigmentlosigkeit der Augen bei Termiten ein typisch larvales Merkmal ist, ist dies wahrscheinlich eine Juvenilhormonwirkung. Dass gerade die juvenil wirkenden C.a. die EG-Produktion stimuliert haben, spricht dafür, dass tatsächlich die Aktivität der C.a. einen Einfluss auf die EG-Kompetenz hat. Es scheint also zwischen der in histologischen Untersuchungen festgestellten Vergrösserung der C.a. nach jeder Häutung und der EG-Kompetenz eine Korrelation zu bestehen.

In allen Versuchen, in denen eine gonadotrope Aktivität der C.a. erwartet werden durfte (IM, PG, EG) traten in grosser Zahl VS auf (Tabelle 3). Die Auslösung der VS-Differenzierung ist also vermutlich eine Wirkung des gonadotropen Hormons der C.a., oder eventuell des stoffwechselstimulierenden Hormons, falls dieses nicht mit dem gonadotropen identisch ist. In Analogie hierzu müssten wir dann auch bei den N_2 vor Imaginalhäutung und bei den S eine Produktion des gonadotropen oder stoffwechselstimulierenden Hormons annehmen, da die C.a. von solchen Spendern ebenfalls VS-Differenzierungen auslösen konnten, bei den N_2 sogar in auffallend hohem Prozentsatz. Die Hemmung der Augenpigmentbildung, die wir als Juvenilhormonwirkung betrachten, war in allen diesen Fällen, mit Ausnahme der C.a. aus N_2, zu beobachten. Es scheint also, dass imaginale C.a. oder solche aus EG, wenn sie in PE oder L implantiert werden, unter dem Einfluss des larvalen Milieus neben dem gonadotropen auch wieder Juvenilhormon produzieren können. Dass adulte C.a. in Larven eine Juvenilhormonwirkung haben können, wurde auch für andere Insekten nachgewiesen (*Dixippus*, PFLUGFELDER, 1940; *Melanoplus*, PFEIFFER, 1945; *Rhodnius*, WIGGLESWORTH, 1948). Bei *Platysamia cecropia* produzieren die C.a. sogar im Adulttier Juvenilhormon, das in Extrakten nachgewiesen werden konnte (WILLIAMS, 1956). Dass die C.a. der N_2 aber anscheinend nicht umgestimmt werden können, scheint uns biologisch sinnvoll zu sein, da sie sonst leicht die normale Metamorphose stören könnten.

Die einzigen Transplantate, die überhaupt keine Wirkung hatten, waren diejenigen der sich nicht in Häutung befindlichen N_2, bei denen auch gar keine Hormonwirkung zu erwarten ist.

Da es also wahrscheinlich ist, dass zwei Hormone der C.a. an der Auslösung der Differenzierung der Kasten beteiligt sind, können wir uns etwa folgende Vorstellung über die Hormonwirkung machen:

Die EG-Kompetenz ist abhängig von der Juvenilhormonsekretion der C.a. Eine Erhöhung der Juvenilhormonwirkung erhöht die Kompetenz. Sind die C.a. juvenil aktiv, so wird durch das Fehlen der Geschlechtstiere bzw. ihrer Pheromone die EG-Differenzierung ausgelöst. Die Faktoren, die hierfür verantwortlich sind, sind nicht bekannt. Sie müssen ihrerseits sehr bald direkt oder indirekt die Prothorakaldrüsen zur Ausschüttung des Häutungshormons anregen.

Es ist möglich, dass die VS-Kompetenz von den gleichen Faktoren abhängig ist wie die EG-Kompetenz, also von der Juvenilhormonsekretion. Die VS-Differenzierung wird aber durch eine Umstellung der C.a. auf die Produktion

von gonadotropem Hormon ausgelöst. Dadurch wird die Differenzierung eingeleitet. Sie wird bei Abwesenheit der Geschlechtstiere beschleunigt. Ist die Differenzierung so weit fortgeschritten, dass sie bei der Auslösung der Häutung abgeschlossen sein kann, so wird direkt oder indirekt die Prothorakaldrüse zur Ausschüttung des Häutungshormons veranlasst.

Mit der Einleitung der VS-Differenzierung wird die EG-Kompetenz stark herabgesetzt. In Einzelfällen kann es aber trotzdem noch zur Auslösung einer EG-Differenzierung kommen. Es entstehen dann Interkasten, bei denen je nach dem Zeitpunkt der Häutung die Soldatenmerkmale mehr oder weniger stark ausgebildet sind.

Diese Vorstellungen über die hormonale Steuerung der Kastendifferenzierung sind nicht bewiesen. Sie widersprechen unseren Befunden nicht, sind aber vorläufig nur als Arbeitshypothesen für unsere weiteren Forschungen aufzufassen.

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ZUSAMMENFASSUNG

(1) In verwaisten Kolonien von *Kalotermes flavicollis* bewirkt die Implantation von Corpora allata aus Nymphen vor der Imaginalhäutung, von Imagines, primären Geschlechtstieren, Ersatzgeschlechtstieren und Soldaten in Pseudergaten und Nymphen eine Hemmung der Ersatzgeschlechtstier-Differenzierung und die Produktion von Vorsoldaten in einem hohen Prozentsatz. Corpora allata aus gewöhnlichen Nymphen und Pseudergaten haben keine Wirkung auf die Entstehung von Soldaten. Corpora allata aus Pseudergaten vor der Häutung haben jedoch eine Erhöhung der Ersatzgeschlechtstier-Frequenz zur Folge. Alle Implantate, mit Ausnahme derjenigen der Nymphen des 2. Stadiums haben eine hemmende Wirkung auf die Pigmentierung der Augen der entstandenen Ersatzgeschlechtstiere.

(2) In normalen, Geschlechtstiere enthaltenden Kolonien hat die Implantation von Corpora allata aus Ersatzgeschlechtstieren in Pseudergaten und Nymphen ebenfalls die Produktion von Vorsoldaten zur Folge. Die meisten Vorsoldaten entstehen aber erst etwa 15–20 Tage später als in den verwaisten Kolonien.

(3) In einigen Versuchen sind Interkasten zwischen Vorsoldaten und Pseudergaten oder Ersatzgeschlechtstieren entstanden. Solche Interkasten können auch dadurch erzeugt werden, dass 10 Tage nach einer Implantation von Corpora allata aus Ersatzgeschlechtstieren Ecdyson injiziert wird. Sie entstehen also dann, wenn die Häutung ausgelöst wird, bevor die Vorsoldaten-Differenzierung abgeschlossen ist.

(4) Durch Schnürungsexperimente konnte gezeigt werden, dass das nach Auslösung der Ersatzgeschlechtstier-Häutung auftretende Wachstum der Corpora allata nichts mit der Ersatzgeschlechtstier-Differenzierung zu tun hat. Nach Abschnürung des Kopfes differenzieren sich die äusseren Ersatzgeschlechtstier-Merkmale normal, dagegen unterbleibt das Wachstum der Oocyten in den Ovarien. Bei der normalen Ersatzgeschlechtstier-Differenzierung setzt also die gonadotrope Aktivität der Corpora allata schon vor der Häutung ein.

(5) Es ist wahrscheinlich, dass 2 Hormone der Corpora allata bei der Differenzierung der Kasten eine Rolle spielen. Das Juvenilhormon scheint die Ersatzgeschlechtstier-Kompetenz zu erhöhen. Das gonadotrope oder stoffwechselstimulierende Hormon scheint für die Auslösung der Soldaten-Differenzierung verantwortlich zu sein.

RIASSUNTO

In *Kalotermes flavicollis* le pseudoergati possono, mutando, diventare reali di sostituzione, soldati bianchi, ninfe, o, infine, restare pseudoergati. Il destino dell'individuo è stabilito da fattori endocrini, che sono a loro volta influenzati largamente da fattori ambientali, quali la abbondanza di nutrimento e, soprattutto, la presenza di feromoni. LÜSCHER ha dimostrato che l'impianto di corpora allata dei reali di sostituzione in pseudoergati mantenute in presenza dei reali della colonia, provoca la loro differenziazione a soldati bianchi, ed ha osservato che i corpora allata di individui che si preparano a mutare a reale di sostituzione, subiscono un notevole ingrossamento immediatamente prima della muta. Con ricerche istologiche l'autore ha dimostrato ancora che i corpora allata, subito dopo le mute larvali, aumentano di volume; dopo le ricerche di SÄGESSER su *Leucophaea maderae*, è ragionevole interpretare tale aumento come un'attività transitoria dei corpora allata, che è contemporanea, nelle termiti, al periodo in cui gli individui hanno la maggior capacità di diventare reali di sostituzione. Perciò si pone il problema se tale capacità non sia dipendente dall'attività dei corpora allata.

Riassunto delle osservazioni. Quando in colonie di *Kalotermes flavicollis* si impiantano, in pseudoergati o in ninfe, dei corpora allata prelevati da ninfe II^ in muta ad alato, da reali di sostituzione, da reali veri, da soldati, da alati, i ricettori, mutando, diventano per la più parte soldati bianchi; ciò è avvenuto in diversa percentuale a seconda delle caste dei donatori. I corpora allata di ninfe II^ non in muta, non hanno manifestato alcuna influenza e così pure i corpora allata di pseudoergati in muta larvale (in quest'ultimo caso tuttavia, sembrerebbe che l'impianto favorisse la differenziazione a reali di sostituzione).

La presenza della coppia reale nella colonia, fa ritardare di 15–20 giorni la comparsa dei soldati bianchi.

I corpora allata trapiantati da qualunque casta provenissero, con la sola eccezione di quelli forniti dalle ninfe non in muta, hanno molto spesso impedito la pigmentazione degli occhi dei reali di sostituzione comparsi.

In alcuni casi, come conseguenza del trapianto, si sono avute delle intercaste, cioè individui che mostravano contemporaneamente alcuni dei caratteri di soldato bianco e dei caratteri di larva, oppure caratteri di soldato bianco accanto a caratteri di reale di sostituzione. Queste intercaste compaiono quando, 10 giorni dopo l'impianto dei corpora allata di reali di sostituzione, si provoca nei ricettori una muta per mezzo di una iniezione di ecdyson. Evidentemente le intercaste compaiono quando la muta avviene prima che sia concluso il processo di differenziazione a soldato bianco.

L'accrescimento dei corpora allata, che nelle mute a reale di sostituzione incomincia appena la larva ha svuotato l'intestino, non è in rapporto al processo di differenziazione della casta, poichè se si isola il capo dal torace e dall'addome per mezzo di una legatura praticata al collo, l'individuo, dopo la muta, presenta le caratteristiche esterne di reale di sostituzione; non avviene invece l'accrescimento degli oociti nei suoi ovari, sicchè bisogna ammettere che l'accrescimento dei corpora allata è in rapporto alla funzione ovarica, e che la loro attività gonadotropa comincia già prima del concludersi della muta.

Dalle osservazioni fatte sembrerebbe che due ormoni prodotti dai corpora allata prendano parte ai processi di differenziazione delle caste. L'ormone giovanile sembrerebbe aumentare negli individui la capacità a diventare reali di sostituzione, forse aumentando la loro sensibilità all'assenza di feromoni. L'ormone gonadotropo o l'ormone che stimola il metabolismo, sembrerebbe invece essere responsabile della induzione della differenziazione a soldati bianchi.

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ACYLPHOSPHATASE IN ORGANS OF A GRASSHOPPER, *ACRIDA BICOLOR* THUNBERG*

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Abstract—Data are reported on the content, heat-resistance, and optimum pH of acylphosphatase in organs of the grasshopper *Acrida bicolor*; results are parallel to those obtained for acylphosphatase in mammalian organs.

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INTRODUCTION

ACYLPHOSPHATASE is a widespread and active enzyme and there is now some evidence (HARARY, 1957a; BACCARI *et al.*, 1960) that one of its functions in the cell is the control of the rate of metabolic processes through the splitting of metabolically formed acylphosphates. Apparently acylphosphatase does not exist as a unique type of enzyme: acylphosphatase first described in mammalian muscle by LIPMANN (1946) is heat-stable at acid reaction (pH 3); but the existence in nature of another different type of enzyme can be suspected by the results of the same writer with kidney, where the activity is diminished by 80 per cent after heating at pH 3. Similar results were obtained by HARARY (1957a) with muscle and liver preparations and were explained by this writer assuming the existence of two types of acylphosphatase, one thermostable and one thermolabile, with the prevalence of the first type in muscle and of the second type in liver. In yeasts GUERRITORE *et al.* (1959a) found an acylphosphatase labile to acidity and to heat.

In the present paper are reported results of an investigation on acylphosphatase of insect organs: quantitative determinations of total and thermostable enzyme were carried out, and activity as a function of pH was studied. Experiments were carried out on organs of a grasshopper, *Acrida bicolor*.

A previous enzymologic research on the same species concerns the existence of a Mg^{++}-activated ATPase in its colleterial glands (GUERRITORE *et al.*, 1958).

MATERIALS AND METHODS

Homogenates of testis, male accessory glands, ovary, colleterial glands, muscle, and intestine were prepared in the Potter apparatus with cold 0·1 M KCl; the homogenate dilution and the quantity added in tests for the determination of

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acylphosphatase activity varied with the different tissues, so as to obtain measures of initial velocity.

The substrate was lithium acetylphosphate, and the enzymatic hydrolysis was determined with the hydroxylamine method of LIPMANN and TUTTLE (1945).

On the basis of KOSHLAND'S definition (KOSHLAND, 1955), the acylphosphatase unit was taken to be that enzyme quantity which in the presence of 6 μmole of acetylphosphate dissolved in 1 ml of acetate buffer pH 5·4, catalyses the hydrolysis with an initial velocity of 0·15 μmole/ml min at 37°C.

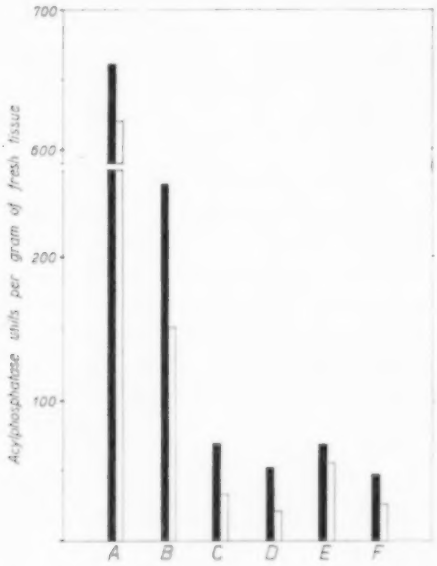

FIG. 1. Content of total and thermostable acylphosphatase in organs of *Acrida bicolor* Thunb. Tests for measuring enzyme units were composed by an homogenate, 12 μmole of acetylphosphate, and 600 μmole of acetate buffer pH 5·4 in a total volume of 2 ml—20 min at 37°C. For determination of heat stability at an acid pH, homogenates were first brought to pH 3·2–3·3 and heated for 10 min at 85°C, then tested at pH 5·4 for acylphosphatase activity as indicated above. A, testis; B, male accessory glands; C, ovary; D, colleterial glands; E, muscle; F, intestine. ■, fresh homogenate; □, homogenate heated 10 min at 85°C.

RESULTS

Results are shown in Figs. 1 and 2. Acylphosphatase is present in all the organs studied, with the highest content in testis; in testis and muscle the enzyme heat-stable at acid pH is largely preponderant. A parallelism can be pointed out between these results and results obtained with mammalian tissues by GUERRITORE *et al.* (1959b). The optimum pH of action, for all the investigated organs, is pH 5, i.e. about the same as that for mammalian acylphosphatase (HARARY, 1957b).

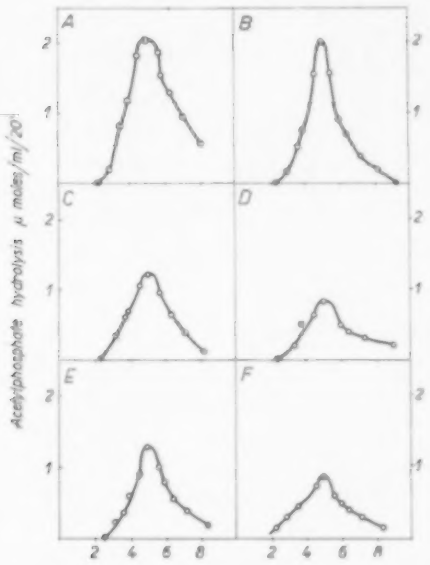

FIG. 2. Acylphosphatase activity as a function of pH in organs of *Acrida bicolor* Thunb. Tests were composed by homogenate, 12 μmole of acetylphosphate and 200 μmole of buffer in a total volume of 2 ml—20 min at 37°C. The homogenate quantity used for curves A and B was respectively a sixth and a half of the quantity used for other tests. Different buffers were used for each pH zone, namely glycine-HCl, acetate, cacodylate or glycine-NaOH buffers; controls for inhibitory effect of glycine or cacodylate were negative. A, testis; B, male accessory glands; C, ovary; D, colleterial glands; E, muscle; F, intestine.

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OBSERVATIONS ON HYPOPUS DEVELOPMENT IN THE ACARINA

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Abstract—The changes that take place in the different organs and tissues during the development of the hypopus result in the hypopus stage enclosing the partly developed organs and tissues of the succeeding deutonymph stage. The form of the hypopus stage depends on the degree of development of the epidermis in the hypopus direction. It varies from the cyst-like form of *G. domesticus* to the mobile form of *H. polypori*, which is utterly unlike the other stages of the life cycle. The cyst-like forms resemble the diapause condition in insects in that they can be terminated by exposure to low temperatures. The mobile form can be initiated and terminated by altering the humidity level of the environment. It is suggested that the potential for hypopus development resides in the epidermis which, in the case of the mobile hypopus, is sensitive to the humidity changes that control hypopus development.

INTRODUCTION

IT has long been known that certain Tyroglyphid mites are capable of developing peculiar resistant hypopus stages. In discussing the production of these hypopi SOLOMON (1943) emphasized the confusion that existed over their development. He commented also on the need for further investigations leading to a better understanding of the factors responsible for modifying the life cycle so as to produce these stages.

Several workers, however, among them POLEZHAEV (1940) and SCHULZE (1924a), had claimed that changes in temperature and relative humidity influenced the number of hypopus forms produced in cultures of *Glycyphagus destructor* Schr. and *Tyroglyphus farinae* L. SCHULZE (1924b) also maintained that a shortage of food induced an increased hypopus production in *Caloglyphus rodionovi*. HORA (1934) concluded that the production of hypopi during the life cycle of *G. domesticus* Deg. was genetically determined. This may be so in some cases, but it was evident that the environment also seemed capable of exerting an influence on the course of the life cycle. In addition, despite the descriptions of the anatomy of the stages, including the hypopus stage, of *G. domesticus* (HORA, 1934) there remained the problem of precisely how the development of the hypopus stage was related to that of the other stages.

The hypopus stages of different species show considerable variation in both design and behaviour. They range from immobile cyst-like forms, through intermediate forms with rudimentary appendages, to extremely mobile forms. These mobile forms differ greatly from the other stages in the life cycle in both structure

and behaviour. Early workers were misled by this and placed them in a separate genus which they named *Hypopus*. This descriptive term has been retained to denote what has been regarded as an extra stage in the life cycle of certain species of mites.

In Tyroglyphid mites, the egg hatches a hexapod larva. This larva develops into a nymph, the protonymph, which in turn is capable of developing either to the deutonymph or to the hypopus stage. Whereas the deutonymph develops directly to the adult, the hypopus first changes to the deutonymph before the adult stage is reached.

Until now, little was known of the factors responsible for controlling the direction of development. It was, therefore, of considerable interest to find, during exploratory investigations on the production of these hypopus forms in different species, that the initiation and termination of the hypopus condition in *Histiostoma polypori* Oud. depended on changes in relative humidity. This provided an excellent example of the extrinsic control of morphogenesis as well as an opportunity of shedding light on the development of the hypopus stage.

The aim of this work, however, has been to investigate the production of cyst-like, intermediate, and mobile types of hypopi in order to determine what the differentiation of hypopus forms entails and to what extent, if any, their initiation and termination is influenced by the external environment.

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MATERIAL AND METHODS

The present work is based on a study of *Histiostoma polypori* Oud., *Glycyphagus destructor* Schr., and *G. domesticus* Deg. These species produce mobile, intermediate, and cyst-like hypopus forms respectively, as shown in Fig. 1.

H. polypori was obtained by collecting the hypopus forms which adhere to the cuticle of the common earwig *Forficula auricularia*. When placed on moist filter paper in Petri dishes the hypopus forms soon change to deutonymphs. The deutonymphs feed readily on decaying insect tissue and change to adults which soon lay eggs. In a short time a thriving colony of all stages has been produced and this can be subcultured as required. *Glycyphagus* spp. were obtained from infested flour and reared on a thin layer of flour in shallow dishes having ground glass edges and fitted glass covers. Each dish had in it one or two thin slices of cork in the crevices of which developing hypopi tended to aggregate, thus aiding their observation and collection. For more detailed observation, mites singly or in small groups were maintained in special culture cells (Jones, 1950). By their use, it was possible to ascertain from changes in structural features whether a protonymph was destined to give rise next to a deutonymph or to a hypopus form.

In all three species studied it was possible towards the end of the protonymphal stage to distinguish a mite which was proceeding towards the hypopus form from one which would become a deutonymph. Mites of *Glycyphagus* spp. developing towards the hypopus could be recognized by a series of markings on the cuticle forming a reticulate pattern in *G. destructor* and series of conchoidal lines in *G. domesticus*. These markings became more pronounced as the moult to the hypopus was approached. In *H. polypori* the markings took the form of a series of dots confined to the dorsal anterior region of the cuticle. Future deutonymphs of all three species showed none of these features.

In the case of *H. polypori*, a culture containing a high proportion of hypopus forms could be obtained by allowing the culture to dry up slowly. Eventually such a culture would be entirely composed of hypopus stages. They survive in low humidity conditions which

are lethal to the other stages, and when the humidity is suddenly raised to saturation level all the hypopus forms readily develop into deutonymphs.

It was therefore possible to predict with reasonable certainty the different steps in the development of these various forms. This also made it possible to obtain serial sections of the three species studied in various stages of normal and hypopial development.

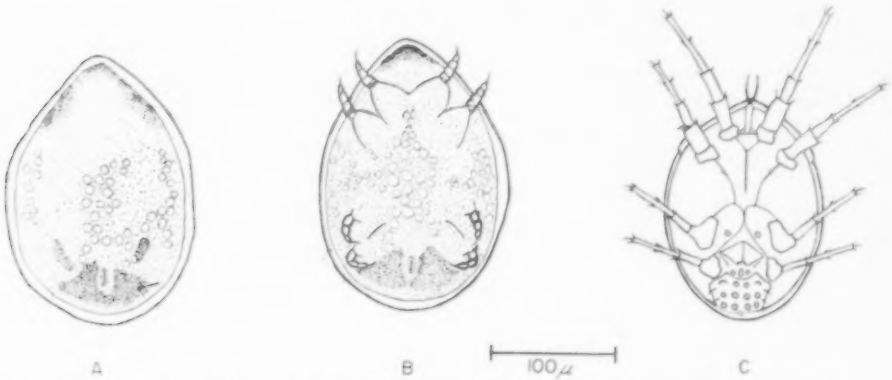

FIG. 1. Hypopus forms. A, cyst-like type of *G. domesticus*. B, intermediate type of *G. destructor*. C, mobile type of *H. polypori*.

For the preparation of serial sections hypopi of *Glycyphagus* spp. were fixed by placing them in cold alcoholic Bouin which was then heated to 50°C and kept at this temperature for 24 hr. Nymphs of *Glycyphagus* spp. and all stages of *H. polypori* were immersed in the fixative at the high temperature. Dehydration was carried out in alcohols heated to 50°C. Benzene was used for clearing, the transfer from alcohol to benzene being done by gradually increasing the concentration of benzene to prevent the collapse of the more fragile stages. The mites remained in pure benzene for 12 hr before impregnation with 56° m.p. wax. The principal stains employed were Ehrlich's haematoxylin and eosine.

HYPOPIAL DEVELOPMENT

The morphology of the cyst-like hypopus of *G. domesticus* was described briefly along with that of the other stages by HORA (1934). Compared with the nymphal stages, the hypopus was mainly in an amorphous state, although the nervous system appeared to be intact.

In the present work, the changes in the different organs and tissues have been described only to an extent sufficient to clarify the views suggested in this paper.

The integument. In *Glycyphagus* spp. there was a thickening of the cuticle of protonymphs which were going to develop into hypopus forms. There was a general thickening in *G. domesticus*, and series of conchoidal markings became visible on the cuticle surface. In *G. destructor* the thickening was confined to the formation of inwardly projecting ridges of cuticle responsible for the reticulate pattern visible on the cuticle surface.

During the moulting process the epidermis divided into outer and inner layers (JONES, 1954). The inner layer retracted considerably to leave a relatively large exuvial cavity. The outer layer of intermediate cells was not continuous but

consisted of single cells spaced at intervals, these cells remaining attached to the shed cuticle. In *G. destructor* the intermediate cells aggregated into the separate compartments of the reticulate-patterned cuticle.

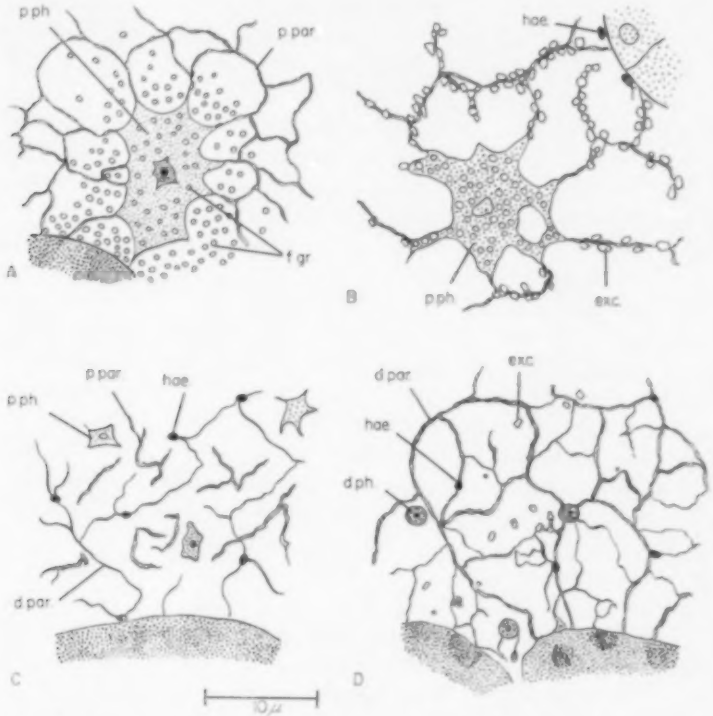

FIG. 2. Sections through the haemocoel of *G. destructor* showing changes that take place during development to the deutonymph through the hypopus stage.

A. Parenchyma network and enlarged phagocyte of protonymph immediately before moult.

B. Disintegrated condition of parenchyma in the hypopus stage.

C. Breakdown of old parenchyma and phagocytes and renewal of parenchyma network by haemocytes during moult of hypopus stage.

D. New parenchyma and phagocytes of newly emerged deutonymph.

p.ph.	phagocyte of protonymph	f.gr.	granules of food reserves
p.par.	parenchyma network of protonymph	hae.	parenchyma-forming haemocyte
ex.c.	excretory crystals	d.par.	new parenchyma network of deutonymph
d.ph.	new phagocyte		

The legs in *G. domesticus* are rudimentary and represented by slightly raised projections. In *G. destructor* all the limb elements are developed in a miniature form, otherwise the hypopus resembles that of *G. domesticus*. In *H. polypori* the epidermis has moulded a mobile hypopus form which is totally unlike the other stages. The dark-brown colour of the cuticle suggests that the tanning process in this hypopus stage is more intensely developed. The spatial relations of the

hypopus are different from those of the other stages, and a unique feature is the development of the battery of sucker discs on the ventral surface at the posterior part of the body.

The haemocoel. The haemocoel contains a parenchyma network which appears to be continuous with the basement membrane of the epidermis and the surface of the gut. There are two types of cells associated with this network, small haemocytes responsible for its formation and large phagocytic haemocytes (Fig. 2 A).

There is a gradual disintegration of the parenchyma during each growth phase and each moult is accompanied by the production of a new network derived from the small haemocytes. These cells produce radiating cytoplasmic strands which condense with those of adjacent cells to form a network.

During hypopus development, however, disintegration of the parenchyma network was not complete, isolated islands of tissue persisting in the haemocoel. It appeared that the phagocytes were concerned with the breakdown of the parenchyma, and in the various hypopi these cells persisted in an enlarged form. They varied both in shape and in size, their nuclei were rounded and stained weakly, and the nucleolus was replaced by a number of granules. The cytoplasm contained a large number of pink-staining granules. The small parenchyma-forming cells were present in relatively large numbers, staining deeply with the stains used, and most had begun to send out radiating cytoplasmic strands (Fig. 2 B). It can be seen, therefore, that in the hypopus, the development of new parenchyma and the final phagocytosis of the old network have come to a halt.

The digestive system. During the growth of each stage the digestive tract undergoes changes, and during the moulting period the gut wall is replaced. Regenerative centres are present as thickened rings composed of fusiform cells with deeply staining nuclei. An oesophageal centre is present at the posterior extremity of the oesophagus. A lateral pair posterior to the oesophageal centre and one at the sphincter region serve the foregut. Each caecum possesses a centre at its extremity. The midgut is regenerated from two centres, one each at the anterior and posterior sphincter regions. The hindgut similarly is reformed from two centres, the dorsally situated part from a centre adjacent to the midgut and the ventral part from a relatively large centre of the epidermis inside the anal aperture.

During hypopus development the disintegration of the old system was completed and the extent of regeneration varied in the three types of hypopi. In *G. domesticus* an arrest of development occurred at an early stage in reconstruction when the prospective tract was composed of strands of cells with deep staining nuclei proliferated from the regenerative centres (Fig. 3 C). In *G. destructor* reconstruction of the gut had advanced further so that a lumen was visible in the various parts of the system. The pharynx and hind gut had secreted a cuticular lining, but an anal opening had not been formed (Fig. 3 B).

In *H. polypori* regeneration of the digestive system reached a still more advanced stage. The mouth opening was formed on the atrophied rostrum, and the hind gut was open at the anus. However, whereas the cells of some parts of the gut

remained small and deeply staining, those of the hind gut developed to their full size and were highly vacuolated (Fig. 3 D).

Musculature. The muscle systems present in the hypopus forms of the three species can be grouped into two series. Firstly, muscle systems which are present in the nymphal stages, and secondly those muscle systems which are peculiar to the hypopus stage, being absent from all other stages.

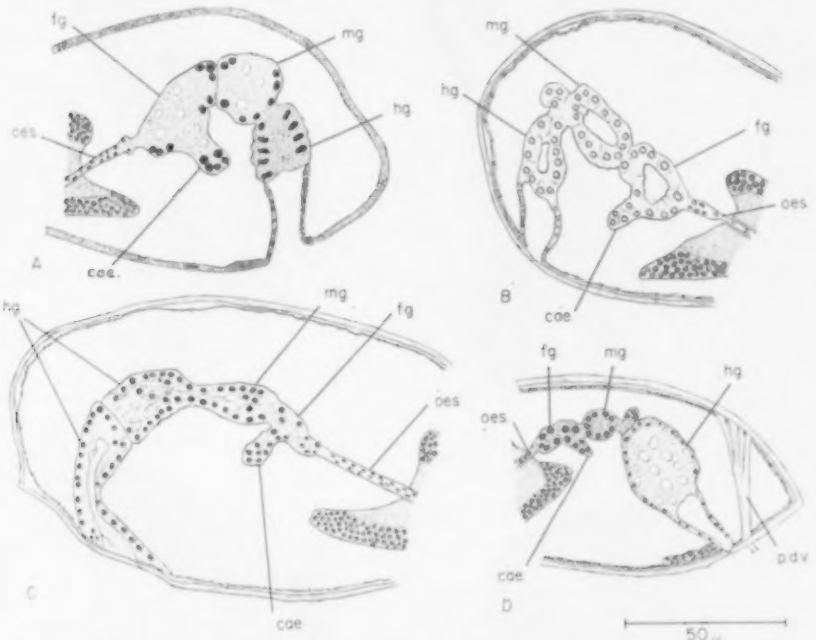

FIG. 3. Sagittal sections of the digestive tract in developing and stable hypopus forms.

A. During hypopus development in *G. destructor*.

B. The hypopus stage of *G. destructor*.

C. The hypopus stage of *G. domesticus*.

D. The hypopus stage of *H. polypori*.

oes.	oesophagus	hg.	hindgut
fg.	foregut	cae.	caecum
mg.	midgut	p.dv.	posterior dorso–ventral muscle

The nymphal muscles in a hypopus stage were present in varying stages of development. Some were present as groups of myoblasts, others as thin strands of myoblast tissue, while certain muscles were fully formed. These observations indicate differential rates of development of the muscle systems in an individual.

The cheliceral retractor muscles of the capitulum and the ventral body muscles in *G. destructor* and *H. polypori* did not disintegrate entirely but persisted with

myoblasts interspersed between the fibres (Fig. 4 B, D). These muscles would be composite ones when fully developed in the deutonymph. In *G. domesticus* these particular muscles were completely replaced, being represented in the hypopus by strands of myoblast tissue (Fig. 4 C). The pharyngeal and anterior

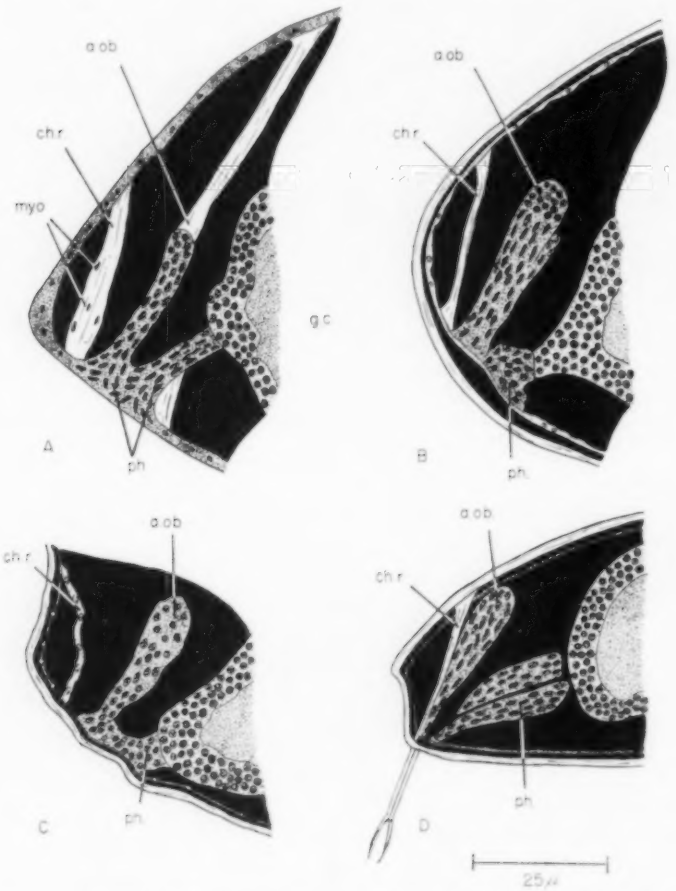

FIG. 4. Sagittal sections showing the muscle systems in the anterior region of different types of hypopus stages.

A. Differentiation of muscle systems during development to the hypopus stage in *G. destructor*.

B. Myoblast tissue in the hypopus stage of *G. destructor*.

C. Hypopus stage of *G. domesticus*.

D. Hypopus stage of *H. polypori*.

ch.r. cheliceral retractor muscle
a.ob. anterior oblique body muscle
g.c. ganglion complex
ph. pharyngeal muscle
myo. myoblasts in tissue of muscle

oblique body muscles in all three species degenerated completely and were represented in the hypopus stages by myoblast tissue (Fig. 4). The dorso–ventral and dorsal muscles as well as the transverse anal muscles were partially broken down and replaced.

The leg muscles of the hypopus forms of both *G. destructor* and *H. polypori* were functional. In *G. domesticus* these muscles were present as groups of myoblasts at the bases of the limb buds.

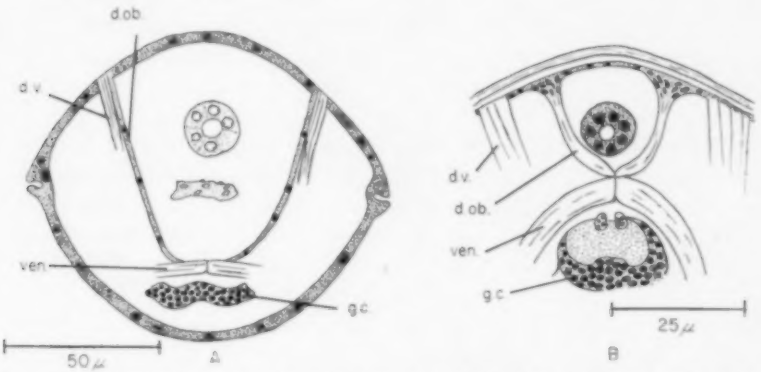

FIG. 5. Transverse sections showing formation of the dorsal oblique muscles during development to the hypopus stage in A, *G. destructor*; B, *polypori*.
d.ob. dorsal oblique muscle d.v. dorso–ventral muscle
ven. ventral muscle g.c. ganglion complex

The hypopial muscle systems which were present only in the hypopus originated as groups of myoblasts during hypopus development. There were two such systems, a posterior dorso–ventral system posterior to the hind gut and a pair of dorsal oblique muscles extending from the dorsal surface to the site where the ventral muscles are attached to the ganglion complex. In all three species, the posterior dorso–ventral muscles were fully formed but they were more extensively developed in *H. polypori* where they were associated with the ventral sucker disc (Fig. 3 D). The dorsal oblique muscles were fully formed in *G. destructor* and *H. polypori* but remained as a thin strand of myoblast tissue in *G. domesticus* (Fig. 5).

Other organs. The salivary glands in normal development are renewed at each moult. During hypopus development the old cells disintegrated, and in the hypopus the glands were represented by lightly staining strands of new tissue.

The reproductive system underwent little change. The only perceptible changes were an increase in size of the genital strands anterior to the gonadial rudiments and their shift to a more vertical position. It seems probable, however, that these changes were a result of the general contraction in size of the mite during the change to the hypopus because the gonadial rudiments remained unchanged.

The central nervous system appeared to be unaltered in the hypopus forms.

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POST-HYPOPIAL DEVELOPMENT

The hypopus stage shed its cuticle during development to the deutonymphal stage. During this process the integument underwent the normal series of changes. This was in contrast to the other systems which simply recommenced their development to give rise to the functional systems of the next stage.

In the haemocoel, the spheres of nutrient material became dispersed and were used up. The parenchyma-forming cells recommenced their activity, sending out further strands of cytoplasm which condensed to form the new network. The phagocytes showed a further increase in size before they themselves disintegrated, being replaced by new rounded deeply staining phagocytes (Fig. 2 C, D) crystals which had accumulated in the haemocoel during the hypopus phase increased further in quantity.

The digestive system and the salivary glands completed their development to become functional organs. In *H. polypori*, the hindgut which had been fully developed in the hypopus only underwent a change in shape to suit the spatial relations of the deutonymph, and the vacuolation of the cells disappeared.

The muscle systems which were specific to the hypopus mite disintegrated completely. The leg muscles of *G. destructor* and *H. polypori* were completely replaced by new nymphal leg muscles derived from regenerative centres at the bases of the legs. In *G. domesticus* the myoblast cells of the rudimentary limb buds present in the hypopus differentiated to form the leg muscles of the deutonymph. The other nymphal muscle systems completed their development.

The reproductive system progressed a little further to assume its normal deutonymphal state. The nervous system remained unchanged except for the extension of nerve branches into the newly formed nymphal appendages.

THE INFLUENCE OF EXTRINSIC FACTORS

In an early work, SCHULZE (1924b) maintained that in a culture of *Caloglyphus rodionovi* a proportion of the nymphs was morphologically distinct from the rest and that these turned into hypopus forms irrespective of further changes in the environment. Of the remaining nymphs, a number could be converted into hypopi by depriving them of food. It is suggested that the nymphs observed by SCHULZE had already acquired the features of those nymphs destined to become hypopus forms, therefore it is not surprising that they were not affected by changes in the environment.

POLEZHAEV (1938, 1940) observed that in a culture of *G. destructor*, the proportion of hypopi increased as the temperature and humidity conditions deviated from those found to be the most favourable for the production of nymphs. The proportion of hypopi did not increase in a culture deprived of food. From a study of *G. domesticus* HORA (1934) concluded that hypopi were produced irrespective of changes in the environment.

Although changes in humidity do not induce hypopus production in *C. rodionovi*, SCHULZE (1924b) found that the hypopus condition of this species could be terminated by increasing the humidity. The mobile hypopus form of

Tyroglyphus farinae also would change back to the nymph by the same means, but, on the other hand, the cyst-like form of this species was unaffected by changes in humidity.

It was evident from these findings that some confusion existed over the question of the relation of hypopus formation to factors in the environment. Moreover, SOLOMON (1943) had directed attention to the need for further controlled experiments. It seems likely, however, that some types of hypopi are more susceptible than others to changes in the conditions of the environment.

The influence of relative humidity

Experiments with *H. polypori* were restricted by the susceptibility of this species to dry conditions. All stages other than hypopi rapidly shrivel up and die if they are deprived of a thin film of moisture in the environment. Hypopi also are unable to survive for long in the absence of free moisture unless they are attached to an earwig.

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When cultures of *H. polypori* were kept moist at temperatures ranging from 12 to 30°C hypopus production was negligible. When the surface film of moisture in the culture was allowed to dry up slowly the production of hypopi increased until the culture consisted almost entirely of hypopus forms. This conversion to hypopus forms took place in the presence of an adequate supply of moistened food and was independent of temperature. Hypopi which were returned to saturated conditions at temperatures within a range from 12 to 30°C all changed to deutonymphs (Fig. 6). By changing the moisture conditions in this way it was possible with few exceptions to switch the development of *H. polypori* to either the nymphal or the hypopial state.

To test the effect of varying the humidity upon hypopus development of *Glycyphagus* spp., mites with a thin film of flour as food were placed in Perspex chambers with inlet and outlet tubes. Air was passed through glycerol–water mixtures to give the required relative humidities (JOHNSON, 1940). Glycerol–water mixtures were prepared to produce relative humidities of 50, 70, 90, and 100 per cent in the air flow. Calcium chloride was used to produce a relative humidity of near 0 per cent. The air was passed through the chamber, which had a volume of 25 cm^3, at a rate of 100 cm^3/hr.

The influence of humidity changes on the development of *Glycyphagus* spp. was much less pronounced than in *H. polypori*. In *G. destructor* there was a slight increase in the proportion of hypopi produced in a culture at the lower humidities and temperatures. However, about 40 per cent of the hypopus forms of this species changed to the nymphal state within 7 days when exposed to saturated conditions at 25°C (Fig. 6). They were unaffected by a humidity of 70 per cent at this temperature.

The hypopus condition of *G. domesticus* could be neither induced nor terminated by bringing about changes in humidity. This seems, therefore, to be in agreement with the view of HORA (1934) that this condition in this species is not affected by environmental changes.

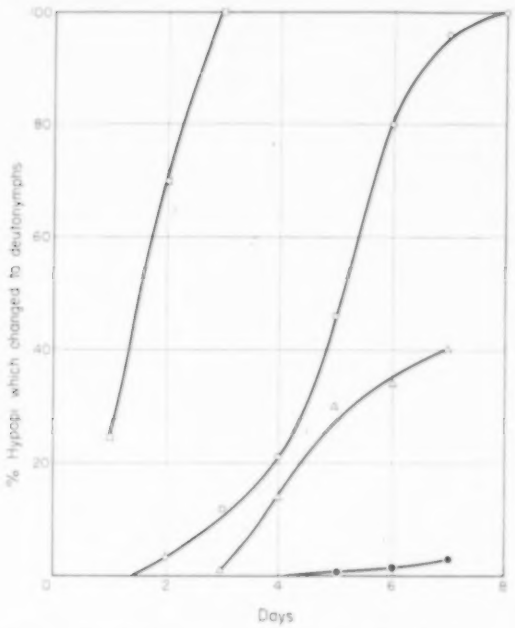

FIG. 6. The effect of exposing hypopi of different species of mites to air saturated with moisture expressed as the percentage which changed to deutonymphs. *H. polypori* at 17°C, ○; *H. polypori* at 25°C, □; *G. destructor* at 25°C, △; *G. domesticus* at 25°C, ●.

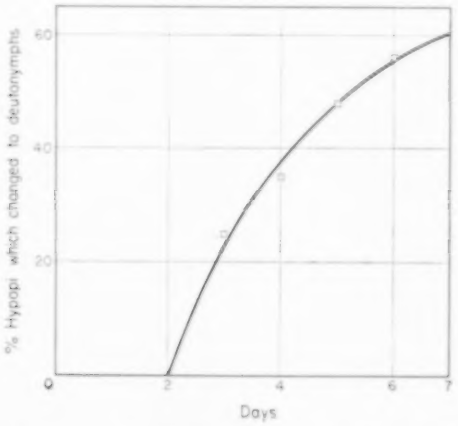

FIG. 7. The effect of exposing hypopi of *G. destructor* to 2°C for 24 hr and thereafter to 25°C with saturated moisture expressed as the percentage which changed to deutonymphs.

The influence of low temperatures

It seemed possible that the hypopus stages of *Glycyphagus* spp. resembled diapause stages of insects and therefore reasonable to suppose that exposure to low temperatures of about 5°C might terminate the hypopus condition. It is well known that such low temperatures can bring about the precocious termination of the diapause state in insects (WILLIAMS, 1952).

In view of this, some exploratory tests were performed on cultures of *Glycyphagus* spp. Hypopi of *G. destructor* were exposed to 2°C 100 per cent r.h. for 24 hr before being returned to saturated conditions at 25°C. Within 3 days 25 per cent of the hypopi had changed to nymphs, 50 per cent of them in 5 days, and over 60 per cent in 7 days (Fig. 7). The percentage of hypopi which changed was therefore higher than in the control when the hypopi were not subjected to the low temperature before being kept at 25°C (Fig. 6). When the exposure to the low temperature was prolonged to 7 days, there was a corresponding increase in the number of hypopi which changed to nymphs.

Hypopi of *G. domesticus* which had been pre-treated at 2°C for 3 weeks showed a significant percentage change to nymphs after 7 days in saturated moisture conditions at 25°C.

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DISCUSSION

The results of this work show that the hypopus condition more or less represents a state in the development towards the next nymphal stage. The hypopus stage is, however, distinct from the other stages in the life cycle, its initiation and termination being marked by a normal moult.

It is characteristic of the hypopus stage that the internal systems, except for certain muscle systems which are present only in the hypopus, are the prospective ones of the following deutonymph stage. The degree of development of these systems depends on the type of hypopus stage produced.

The distinctive qualities of the hypopus would seem to depend on the degree to which the epidermis moulds a form different from that of the other stages. In the case of *G. domesticus* a simple cyst-like hypopus form is produced; in *G. destructor* on this shield-shaped cyst rudimentary appendages are formed. Finally, in *H. polypori* the epidermis moulds a complex shield-shaped form with powerful anterior pairs of legs which bears no resemblance to the other stages. The cuticle of the hypopus is hardened and brown in contrast to the soft cuticle of the other stages. The development of the large number of sucker discs is also a distinctive feature of this hypopus form. Its behaviour and quick locomotory movements are also in marked contrast to the sluggish movements of the nymphal and adult stages.

The development of the hypopus stage into the succeeding deutonymph stage must obviously be accompanied by a moult. This moult is most necessary in the case of *H. polypori*.

It is tempting to suggest that the hypopus condition of *Glycyphagus* spp. resembles the diapause condition of insects. The evidence that changes of humidity

exert far less influence on this condition in these species than exposure to low temperatures tends to support this view. The hypopus condition of *H. polypori*, however, cannot be explained in the same way.

The hypopus form of *H. polypori* would seem to owe its distinctiveness to the capacity of the epidermis to develop rapidly in a direction which ends in the moulding of a form which encloses internal systems most of which are incompletely developed. When the hypopus stage moults, the hypopus mould or cuticle is shed, and the epidermis reverts to moulding the nymphal form to house the enclosed nymphal systems which continue their development.

It is suggested that the potential for switching to hypopial or nymphal development lies in the epidermis. An important fact which has emerged from this work is that development in one or other of these directions can be controlled by changing the humidity conditions. This provides an example of the extrinsic control of morphogenesis.

It is suggested that in the epidermis of *H. polypori* there reside three morphogenetic mechanisms. They are nymphal, hypopial, and adult. Whereas it is reasonable to suppose that development in the nymphal or adult direction is under hormonal control as in insects, it is clear that whether the first nymph develops into a second nymph or into a hypopus condition depends upon humidity. Thus it has been shown that the development of a complex multicellular animal can be switched to either one of two directions by simply altering the humidity levels.

SUMMARY

(1) The hypopus stage encloses the partly developed systems of the succeeding deutonymph stage. Also present are some muscle systems peculiar to the stage.

(2) The form of the hypopus stage depends on the degree of development of the epidermis in the hypopial direction. It may be cyst-like as in *Glycyphagus* spp., or as in the case of *H. polypori* a mobile form totally different from that of other stages.

(3) The initiation and termination of the hypopus stage is marked by a moult.

(4) The hypopus condition of *Glycyphagus* spp. resembles a diapause. It can be terminated by exposure to low temperatures, but not by changes in humidity.

(5) The initiation and termination of the hypopus condition of *H. polypori* can be controlled by altering the humidity levels.

(6) It is suggested that the potential for hypopus production resides in the epidermis. In *H. polypori* the epidermis is sensitive to humidity changes that can control hypopus development.

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THE FINE STRUCTURE OF THE MOSAIC MIDGUT EPITHELIUM OF BLOWFLY LARVAE

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Abstract—By means of electron micrographs many differences in fine structure were demonstrated between the lipophilic and cuprophilic cells which are intermingled to form the mosaic midgut epithelium of *Lucilia* larvae. These two cell types have different functions and a different embryological origin.

The lipophilic cells, which are characterized by large inclusions of storage fat, have a well-developed striated border of unusual structure, being formed of lamellae instead of microvilli. Mitochondria occur fairly evenly throughout the cytoplasm, which also contains scattered membranes of endoplasmic reticulum and fairly numerous small vesicles. Some vesicles bounded by smooth membranes occur in groups and possibly represent the Golgi complex.

The cuprophilic cells have sparse, squat microvilli on their lumen surface. They are also distinguished by the much-infolded basal cell membrane which extends apically for about four-fifths of the height of the cell. Mitochondria occur in great numbers in association with the infolded membranes and much less frequently in the apical region of the cell. Lipoid masses are absent and no Golgi elements were distinguished.

INTRODUCTION

A REMARKABLE pattern of functional and morphological differentiation exists in the midgut of blowfly larvae. Histochemical studies have shown that anterior, mid, and posterior regions may be readily distinguished and, within the mid midgut, five distinct zones are clearly evident. Of particular interest is the second zone of the mid midgut, a mosaic of two cell types, which forms the subject of this paper. One of the cell types—the lipophilic cell—is packed with lipoid spheres and glycogen, contains a cytoplasmic acid phosphatase, and has a distinct striated border. The other cell type of the mosaic—the cuprophilic cell—accumulates copper and iron and possesses esterases, cytochrome oxidase, and dehydrogenases. Glycogen and lipoid are absent, acid phosphatase is weak, and there is no distinct striated border (WATERHOUSE, 1940, 1945; WATERHOUSE and STAY, 1955). The lipophilic cells with their distinct striated border arise embryologically as typical midgut cells from the midgut rudiments. On the other hand, there is strong circumstantial evidence that the cuprophilic cells, which appear to lack a typical striated border, arise from the residual pole cells which are left behind at the posterior end of the egg when some of their number migrate inwards to form the germ cells of the gonads (POULSON and WATERHOUSE, 1958). These residual pole cells move along the dorsal furrow of the developing embryo, are

carried into the interior at gastrulation and are believed to take up their position in the mid midgut epithelium as the anterior and posterior midgut rudiments grow together (POULSON, 1947, 1950).

The present study was undertaken to determine the fine structure of these two mid midgut cell types and to provide further information on their functional differentiation.

It may be important to note that, in *Lucilia*, as in other Diptera, the individual midgut epithelial cells survive for the entire larval life. These cells are very active and grow progressively in size throughout each instar, but they do not divide and do not give rise to the adult midgut. In this way, at least, they differ from the midgut epithelium of most insects in which cell division is continuous and worn-out cells are cast off into the lumen. It is not known whether there are significant structural differences between these two types of epithelium.

MATERIALS AND METHODS

Almost fully-fed third-instar larvae of the Australian sheep blowfly *Lucilia cuprina* (Wied.) were used. These were reared at 30°C under sterile conditions on an artificial medium (WATERHOUSE and STAY, 1955). Determinations on their haemolymph gave values for freezing point depression ranging from — 0·84 to — 0·90°C. Of the various fixatives and tonicities tried, the most satisfactory was ice cold, buffered (pH 7·2), 1 per cent osmic acid solution (RHODIN, 1954) with sodium chloride added to give a freezing point depression of — 0·86°C. After fixation for 3–4 hr, washing in saline and dehydration in gently graded alcohols, the tissues were infiltrated with partially polymerized butyl methacrylate, which was subsequently hardened at 60°C. Sections were cut with glass knives on a Farrant–Powell microtome* (FARRANT and POWELL, 1958) and examined in an RCA EMU-3B electron microscope.

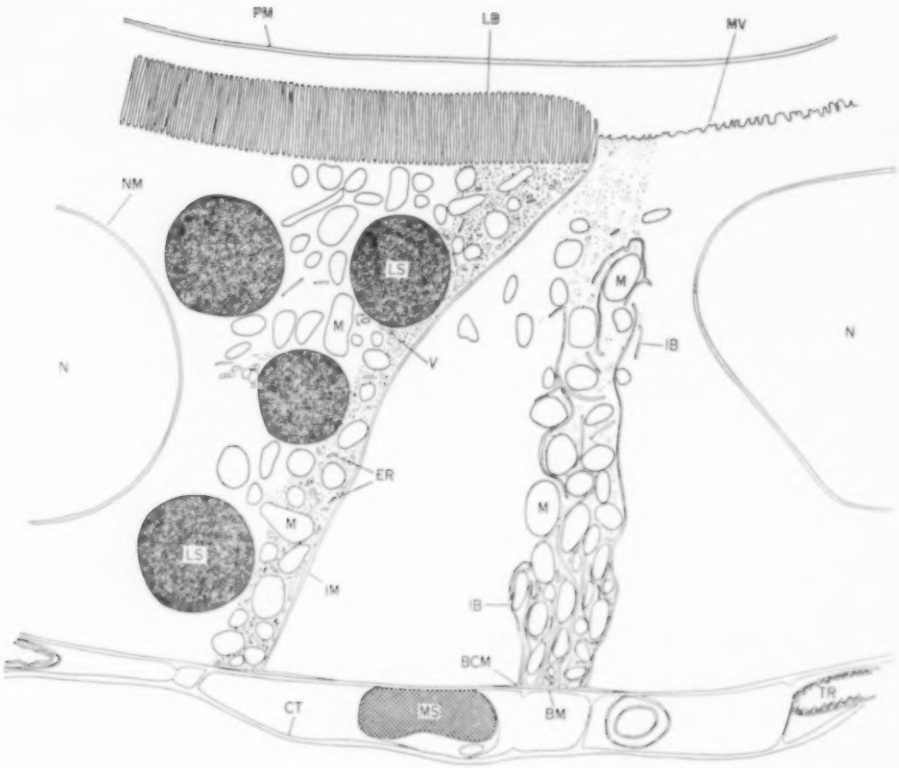
OBSERVATIONS

The two types of cell in mid midgut zone II were readily distinguished in micrographs and their principal features are shown diagrammatically in Text Fig. 1.

(a) *The lipophilic cells*

(i) *Striated border*. These cells have a distinct striated border composed of lamellae about 0·06 μ wide and 1–2 μ (mostly about 1·3 μ) high (Figs. 1–3, 6). The border is clearly not composed of microvilli, for circular or elliptical profiles expected in transverse or oblique sections of individual rodlets were never seen in the many sections examined, although these are a characteristic feature of most sections of other regions of the midgut. Furthermore, sections through the base of the striated border parallel to the surface of the lumen (Fig. 3) clearly demonstrate the undulating arrangement of the lamellae. This takes the form of one or more pronounced curves throughout their length. Branching of the lamellae is

* SI-RO-FLEX Ultramicrotome, Fairey Aviation Company of Australasia, Salisbury, South Australia.



Lipophilic cell	Cuprophilic cell
Lipoid spheres	Copper
Glycogen	(Iron)
Acid phosphatase	Potassium
	Dehydrogenases
	Cytochrome oxidase
	Esterase
Lamellar border	Microvillar border
Simple basal membrane	Infolded basal membrane

TEXT FIG. 1. Diagram of a lipophilic (left) and an adjoining cuprophilic (right) cell from the mid midgut of a *L. cuprina* larva. BCM, basal cell membrane; BM, basement membrane; CT, connective tissue; ER, endoplasmic reticulum; IB, infolded basal membrane; IM, intercellular membrane; LS, lipoid sphere; LB, lamellar border; M, mitochondria; MS, muscle; MV, microvillar border; N, nucleus; NM, nuclear membrane; PM, peritrophic membrane; TR, trachea; V, vesicles.

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Explanation of plates

Abbreviations used in the figures

BCM	Basal cell membrane	LB	Lamellar border
BM	Basement membrane	LS	Lipoid sphere
C	Cuprophilic cell	LU	Lumen of midgut
CT	Connective tissue sheath	M	Mitochondria
ER	Endoplasmic reticulum	MV	Microvilli
G	Vesicles possibly constituting the Golgi complex	P	Ribonucleoprotein particles
		PM	Peritrophic membrane
IB	Infolded basal membrane	TB	Thickening of cell membrane
IM	Intercellular membrane	TR	Trachea
L	Lipophilic cell	V	Vacuole

Except where otherwise indicated the line markers represent one micron

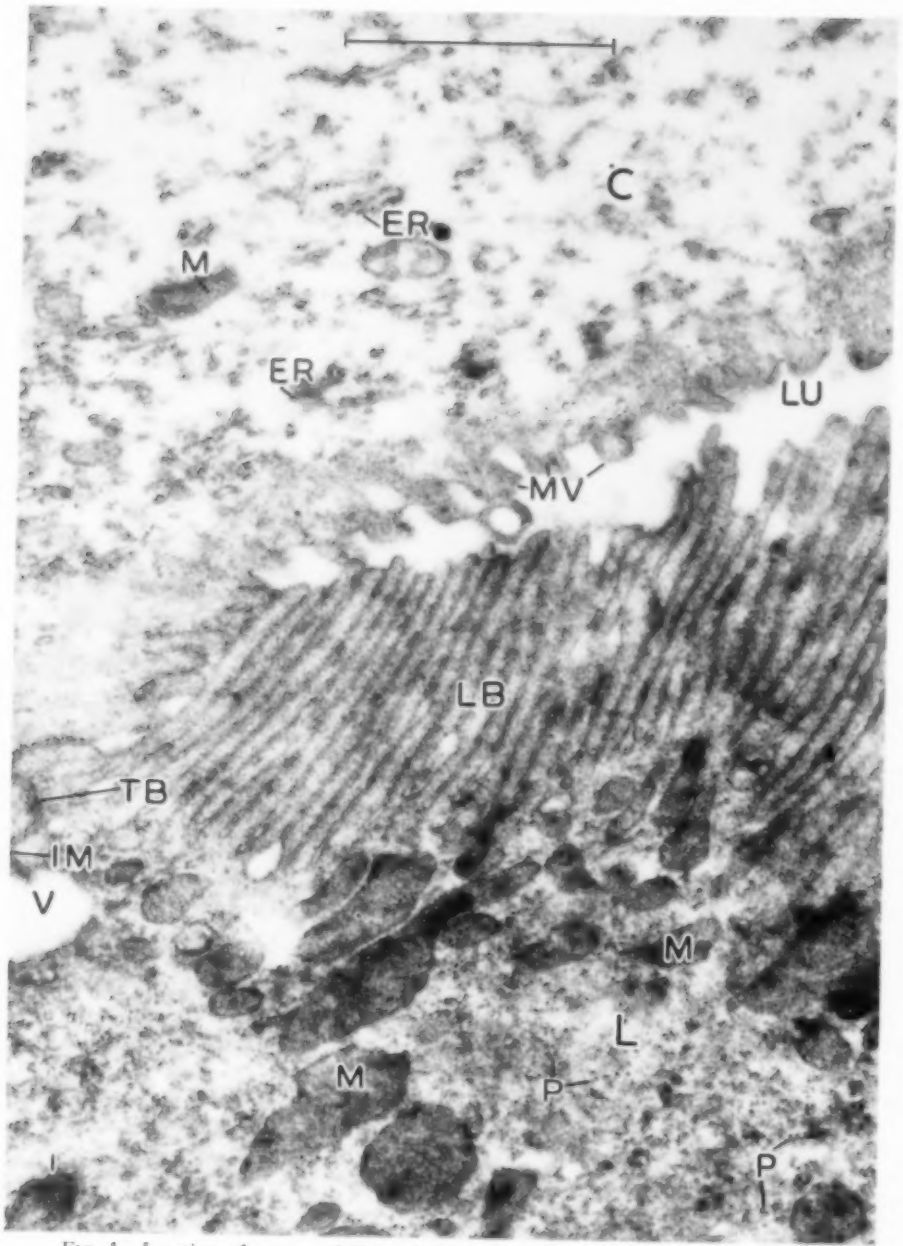

FIG. 1. Junction of a cuprophilic and a lipophilic cell at the gut lumen (LU). The cuprophilic cell (C) has a few profiles of endoplasmic reticulum (ER) and squat microvilli (MV). The lipophilic cell (L) is bounded, on its lumen surface, by a lamellar border (LB) and contains many mitochondria (M) and ribonucleoprotein particles (P). The cytoplasm of the lipophilic cell is much denser than that of the cuprophilic cell. The intercellular membrane (IM) shows a region where the intercellular space is enlarged into a vacuole (V). In this section and in some others there is, near the lumen, a thickening of the membrane (TB). (× 37,800)

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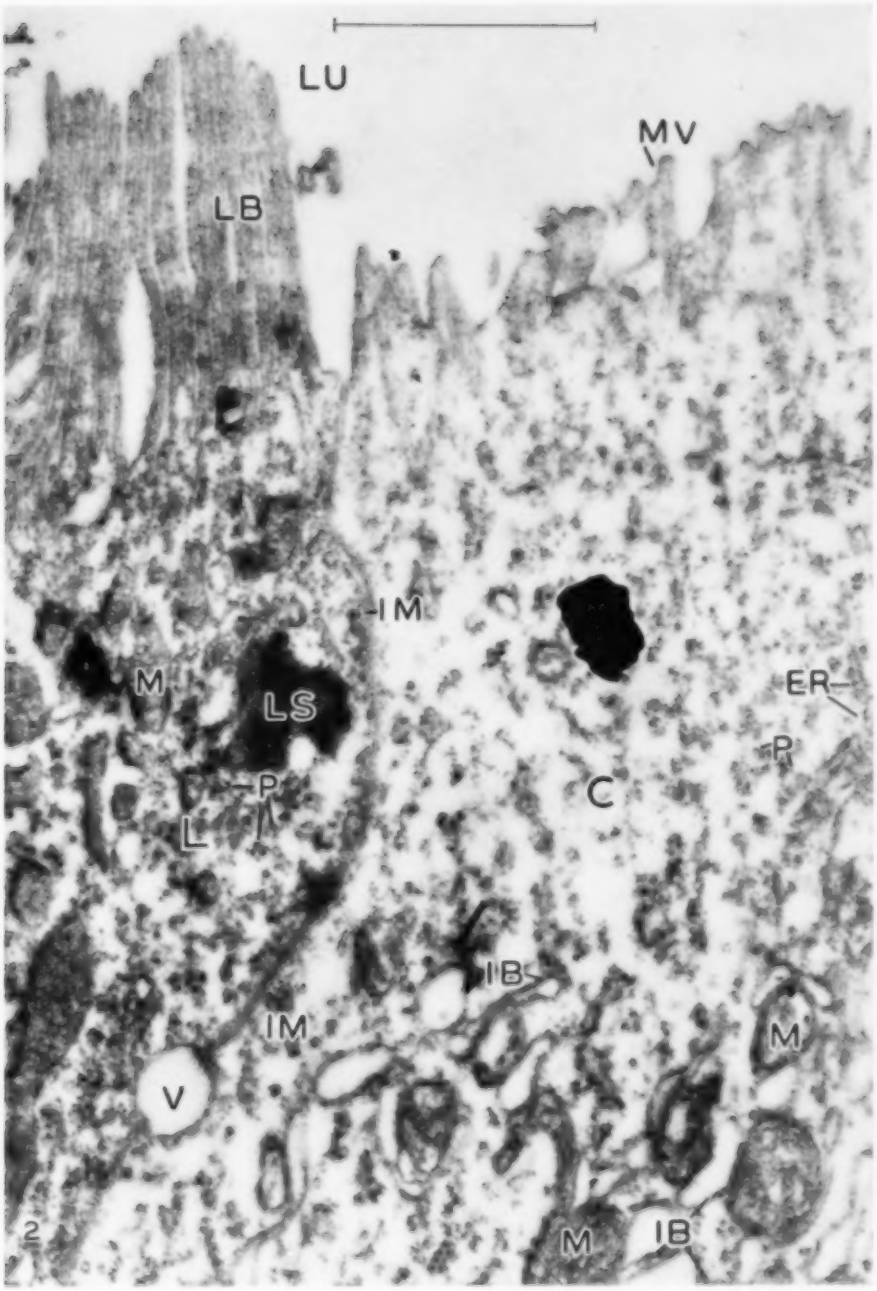

FIG. 2. Junction of a cuprophilic and a lipophilic cell. In the lipophilic cell a body (LS), probably lipoid in nature, is seen. In the cuprophilic cell the upper limit of the smooth membrane system (IB), which extends from the base of the cell, is seen associated with mitochondria (M). The apical region of the cytoplasm is relatively empty of mitochondria and other electron-dense structures. The dense body on the cuprophilic cell is probably a dust particle. (× 36,900)

FIG. 3. Section nearly parallel to the lumen surface of a lipophilic cell and passing close to the base of many lamellae. No elliptical or circular profiles of lamellae are present. The way in which the lamellae run in an undulating fashion for considerable lengths along the cell surface is readily seen. Some bends and junctions are indicated by arrows. A small portion of the lipophilic cell cytoplasm (L) is seen. Two lamellae arising from the same elevated portion of cytoplasm are indicated by asterisk. The lamellar cytoplasm is moderately dense, appears uniform in texture and lacks any organelles or ribonucleoprotein particles. (× 40,800)

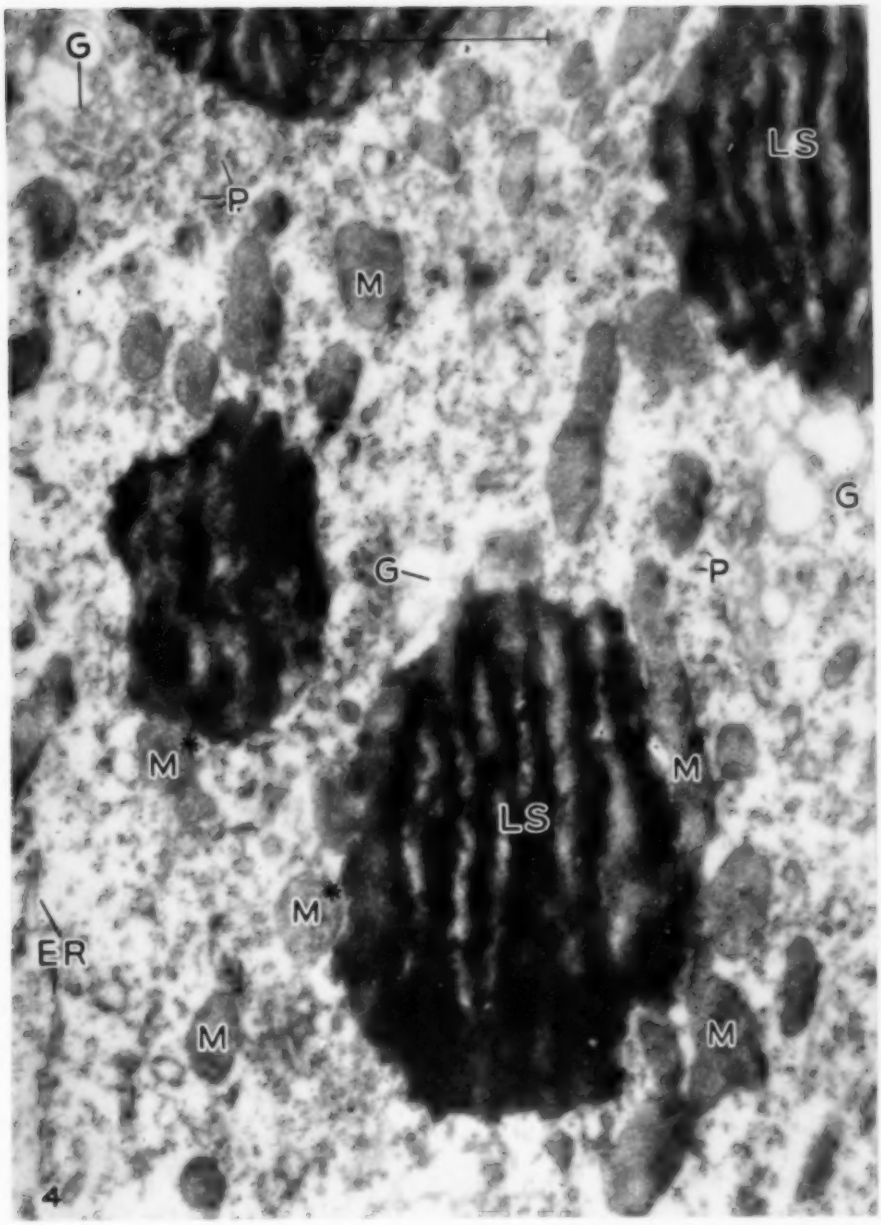

FIG. 4. Cytoplasm of a lipophilic cell with lipoid sphere (LS). The rippled appearance of the lipoid spheres is a cutting artefact. As well as rough membranes of endoplasmic reticulum (ER), there are a few small vesicles surrounded by smooth membranes (G), which may represent Golgi regions. At M* mitochondria are closely apposed to the surface of lipoid spheres. (× 37,800)

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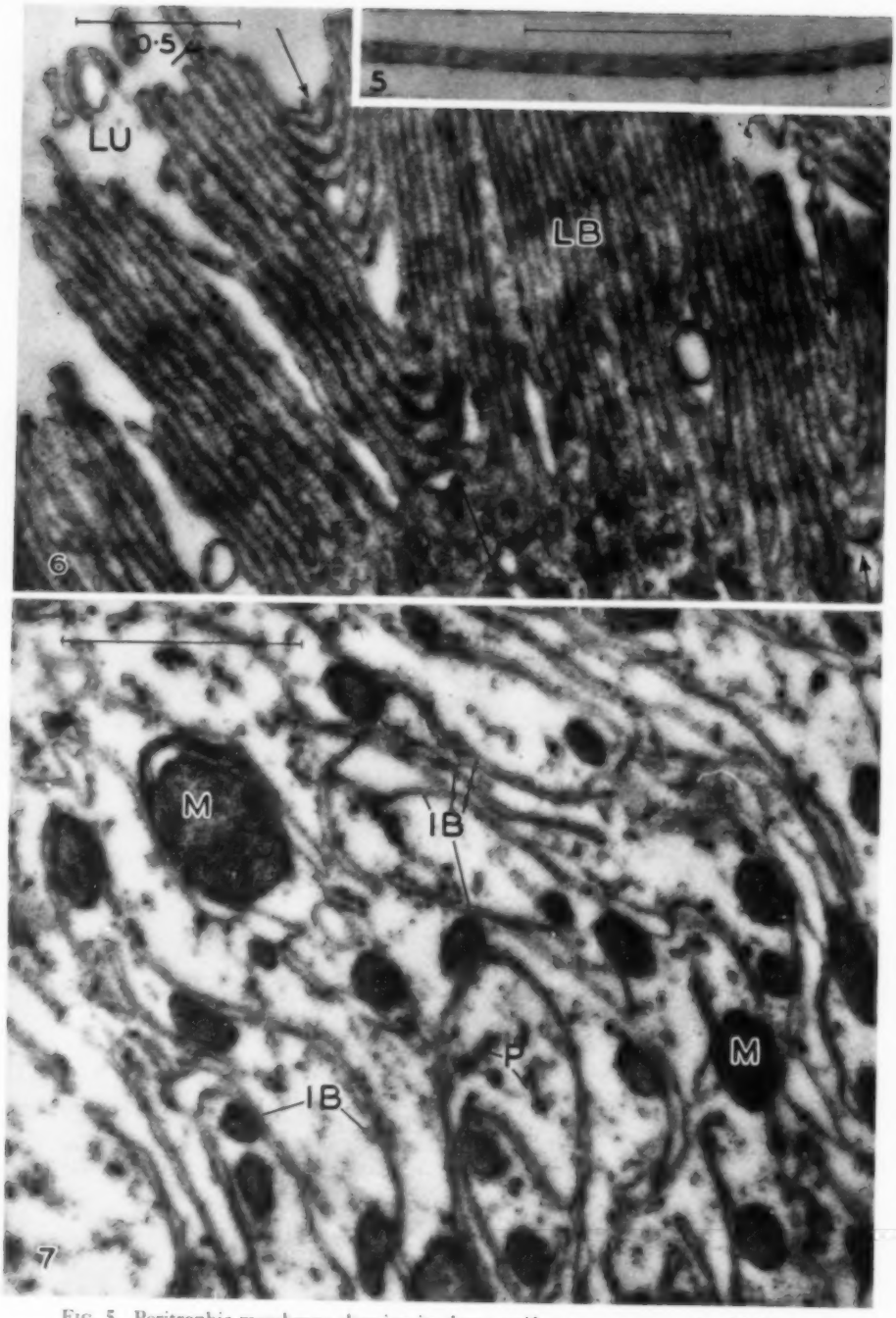

FIG. 5. Peritrophic membrane showing its dense uniform appearance. (× 30,000)

FIG. 6. Portion of a lipophilic cell adjoining the gut lumen. Most of the lamellae have been cut transversely but, at the lines marked by arrows, the lamellae have been cut obliquely and their lamellar nature is shown. (× 46,800)

FIG. 7. The infolded membranes (IB) of the cuprophilic cell near their furthest extension into the cell. Many mitochondria are in the folds of this labyrinth. (× 34,800)

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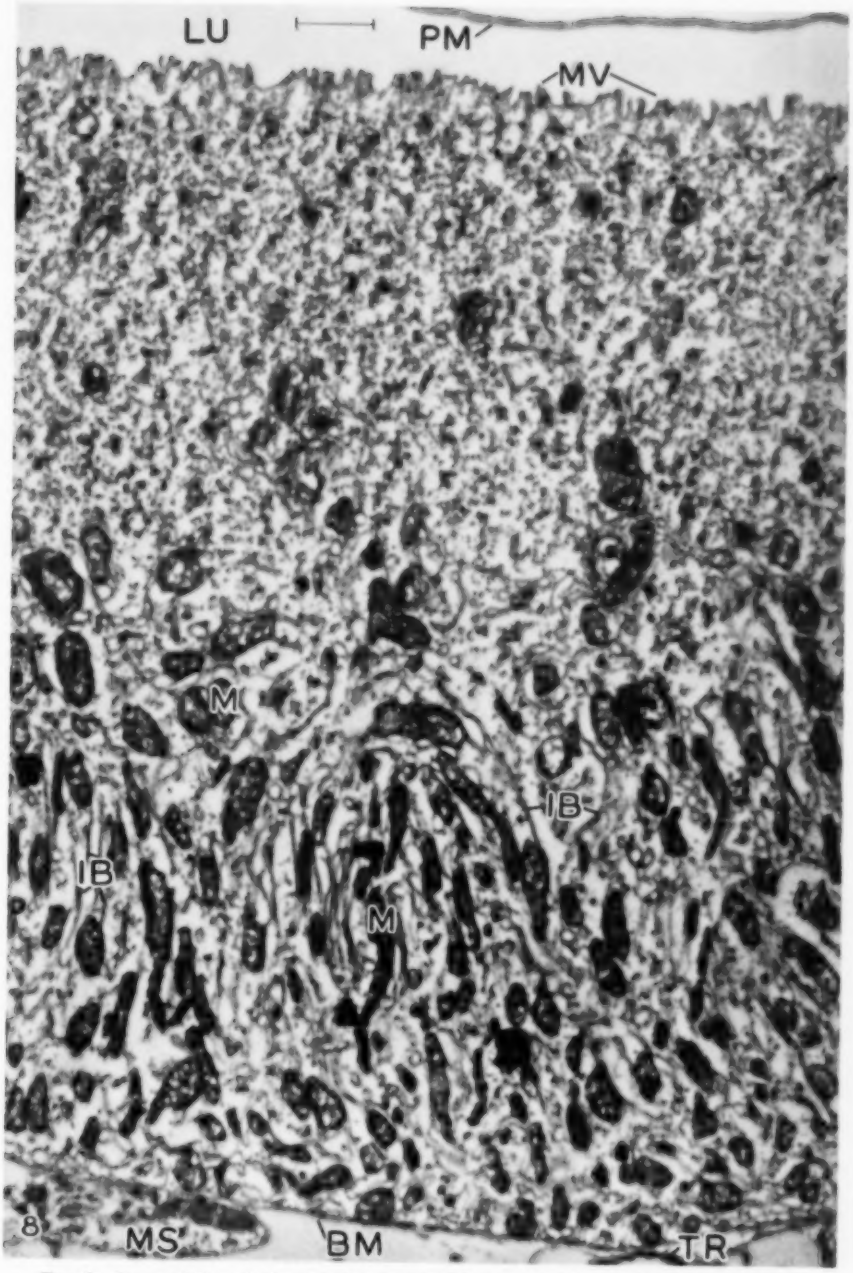
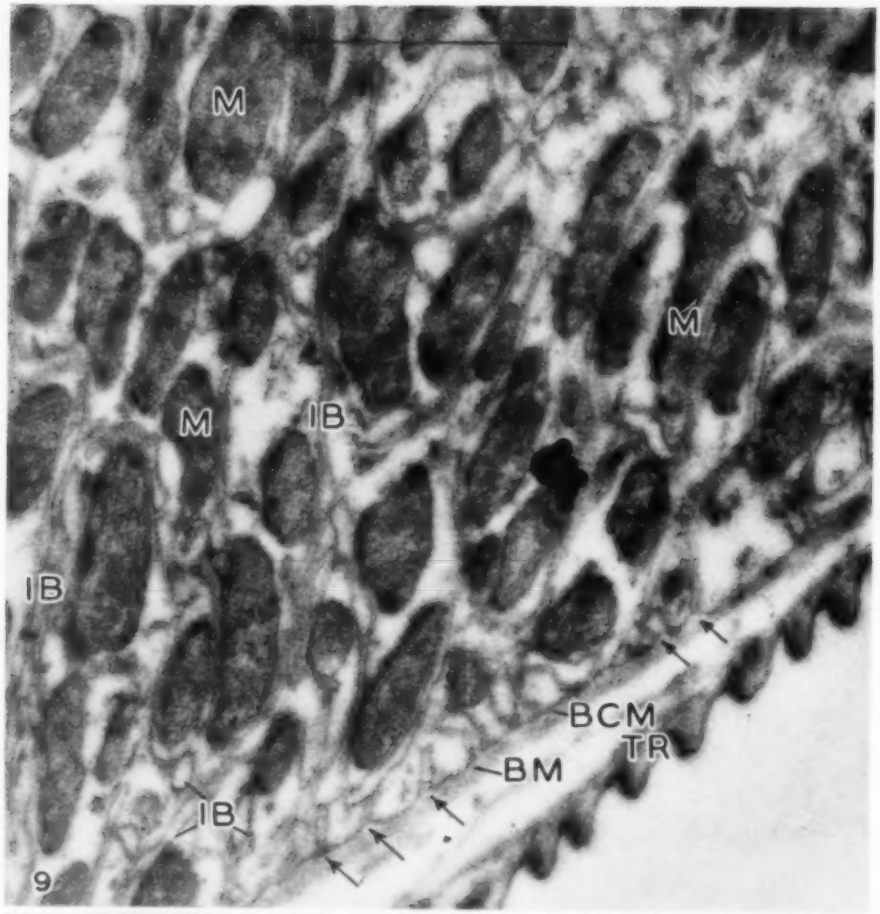


FIG. 8. Cuprophilic cell showing the extent of the system of smooth infolded membranes which are continuous with the basal cell membrane. Mitochondria are located in the basal two-thirds of the cell and are associated with these infolded membranes. The lumen surface is covered with squat irregular microvilli (MV) and, in the lumen, a portion of the peritrophic membrane is visible. Outside the basement membrane (BM), muscle (MS) and trachea (TR) are present. (× 11,400)



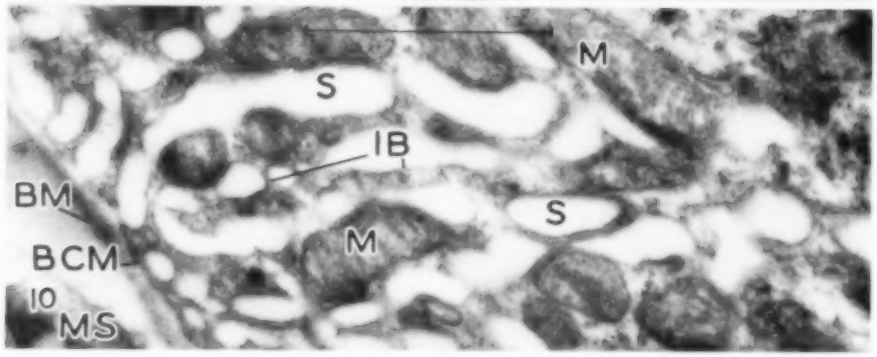


FIG. 9. Basal region of a cuprophilic cell showing continuity (at arrows) of basal cell membrane and infolded membranes. Numerous mitochondria are present between the infolded membranes (IB), which form a very complex labyrinth. On the other side of the basement membrane (BM) is a portion of a trachea (TR). (× 37,800)

FIG. 10. Basal region of a cuprophilic cell in which the space (S) between the infolded membranes is distended, presumably by an aqueous solution. The spaces of an adjacent cuprophilic cell were not distended. (× 34,800)

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FIG. 11. A section of the apical part of a cuprophilic cell showing the squat irregular microvilli covering the surface. The electron-density or staining of the cytoplasm of the microvilli and of the first 0·5μ below the cell surface is greater than that of the rest of the cytoplasm. The furthest apical extent of the infolded membranes and their associated mitochondria is seen in the lower part of the plate. In mitochondrion (M1) the cristae are curved and parallel to one surface. In mitochondrion (M2) the cristae appear as concentric rings. This appearance would be given by a mitochondrion similar to (M1) if the section were in a plane perpendicular to the paper and in a direction indicated by the two arrows. (× 42,900)

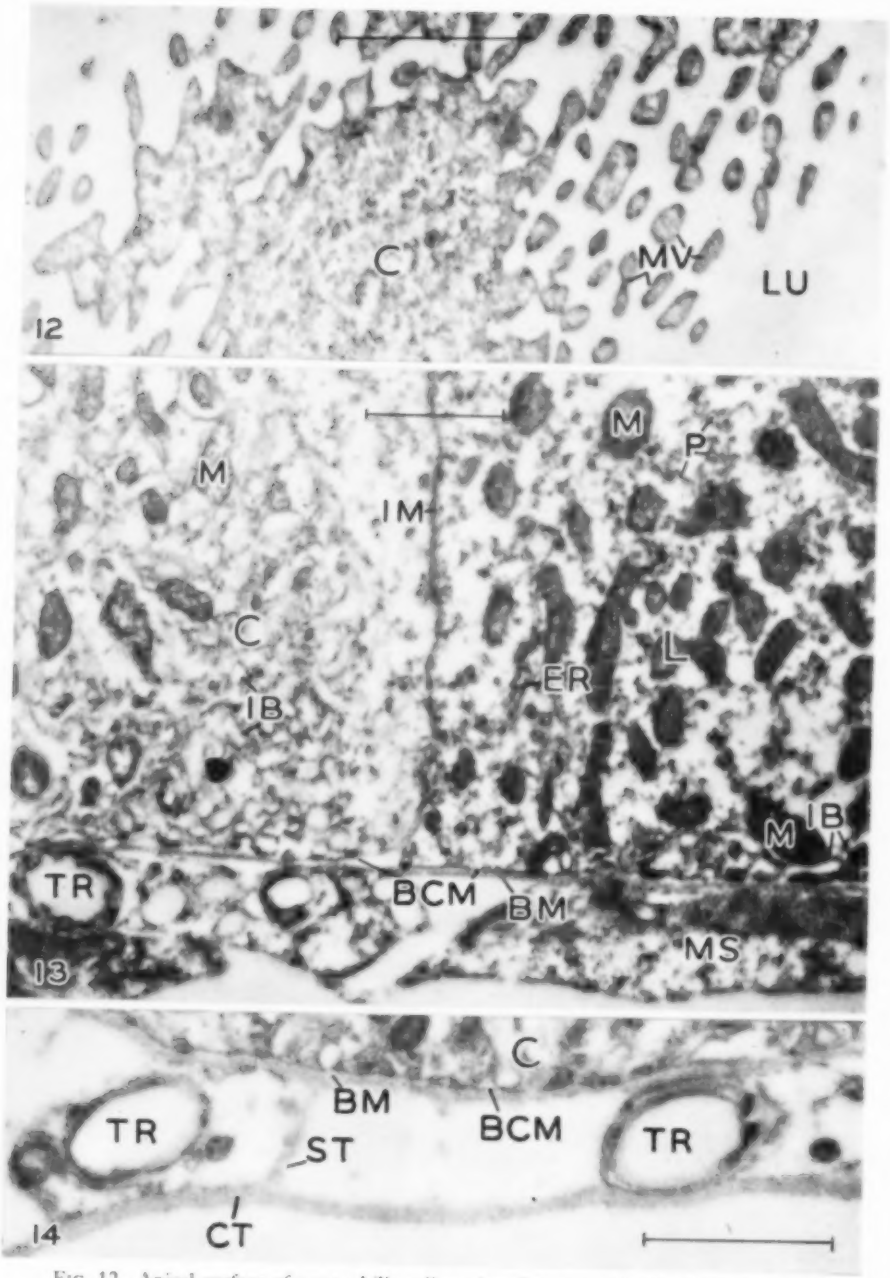

FIG. 12. Apical surface of cuprophilic cell sectioned tangentially showing cross sections of the squat irregular microvilli. (× 25,500)

FIG. 13. Base of adjoining cuprophilic and lipophilic cells. In the cuprophilic cell there are numerous smooth membranes (IB). In the lipophilic cell there is a very limited infolding of the basal membrane (IB) and a few rough membranes (ER) are seen. Outside the basement membrane tracheae (TR) and muscle (MS) are present. (× 19,500)

FIG. 14. Section showing apparently structureless basement membrane (BM) and connective tissue sheath (CT) connected by a strand (ST) of similar material. In the space between the basement membrane and the connective tissue sheath are two fine tracheae (TR). (× 26,400)

occasionally observed (Fig. 3), but this is probably near the base of adjacent lamellae which have a common origin on an elevated portion of the cell surface (Fig. 3). No pores or discontinuities were observed in the cell membrane surrounding the lamellae. The material within the lamellae is fairly dense, uniform in texture, and lacks any cellular organelles.

(ii) *Lipoid spheres.* Lipoid inclusions of various sizes (diameters ranging up to about 4 μ) with a strong affinity for osmic acid and distributed throughout the cytoplasm are a distinctive feature of the cell (Fig. 4).

(iii) *Mitochondria.* These occur fairly evenly throughout the cytoplasm, and no unusual features were noted. They have three-layered surface membranes and cristae which usually appear to be continuous across the mitochondria and to be oriented roughly at right angles to the long axis. In some cells many of the lipoid spheres have one or more mitochondria closely apposed to their surface (Fig. 4) (LEVER, 1957; PALADE and SCHIDLOWSKY, 1958).

(iv) *Nucleus.* The nuclei display no novel features. The double nuclear membrane is 'perforated' by numerous 'pores' as in other insects (ANDERSON and BEAMS, 1956; BAHR and BEERMANN, 1954; GAY, 1955, 1956). In *Lucilia* these pores are about 430 Å in diameter, are surrounded by an electron dense zone 170 Å wide, and are spaced 800–1000 Å from centre to centre. The nuclear sap is rather uniform with small dense granules and fine fibrils. Dense nucleoli also occur.

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(v) *Cytoplasm.* The cytoplasm is fairly dense, and small dense granules (110–150 Å diameter), presumed to be ribonucleoprotein (PALADE, 1955), are scattered generally, although not entirely at random throughout it, giving the cytoplasm a textured appearance. Glycogen is known to occur in these cells but no areas are found that at present can definitely be assigned as the glycogen containing regions. It is probably distributed generally throughout the cytoplasm and, as it is of low electron density, it may be present in regions not occupied by other cellular organelles. On the other hand it may have been extracted from the tissues during dehydration and embedding.

(vi) *Intercellular membrane.* Where cells adjoin (Figs. 1, 2), the cell membrane appears as an opaque layer about 80 Å wide separated from the membrane of the adjacent cell by a less dense region of fairly constant width (about 80 Å). At one or two places in some sections, the zone between the membranes is enlarged to form an oval space (Figs. 1, 2). It is not known, however, whether this is an artefact. Thickenings of closely apposed intercellular membranes near the lumen border are sometimes seen (Fig. 1).

(vii) *Basal cell membrane.* This is about 80 Å wide and is closely invested by the basement membrane. It is generally simple, although very limited infolding is occasionally observed (Fig. 13).

(viii) *Intracellular membranous structures.* Membranes of endoplasmic reticulum are scattered throughout the cell (Fig. 4). Granules, 110–150 Å in diameter, of ribonucleoprotein are attached to these membranes and also lie free in the cytoplasm.

Vesicles containing low density material and surrounded by smooth membranes are fairly numerous. Similar structures are sometimes seen in groups associated with the lipoid spheres (Fig. 4) and these may represent the Golgi complex if, indeed, it is present in these cells.

(b) *The cuprophilic cells*

The fine structure of these cells is immediately distinguished from that of the lipophilic cells by a number of features, notably the absence of a high striated border, by a lower opacity of the cytoplasm, by the much infolded basal cell membrane, and by the infrequent occurrence of mitochondria in the apical fifth of the cell.

(i) *Lumen border.* The cell surface bounding the gut lumen bears squat microvilli (Figs. 1, 2, 8, 11) which are not as closely packed as the lamellae of the striated border of the lipophilic cells. They are irregular in cross-section (Fig. 12) but, to first approximation, are cylindrical and are about 0·09 μ wide and 0·18–0·3 μ high. Their bounding membrane, a portion of the cell membrane, is 100–125 Å wide. The cytoplasm within the microvilli and for a distance of about 0·5 μ below them is denser than that in the remainder of the cell, being similar to that of the lamellae of the lipophilic cells (Figs. 1, 11); it is uniform in texture.

(ii) *Basal cell membrane.* This is about 80 Å wide and is closely invested by the basement membrane. The basal cell membrane differs markedly from that of the lipophilic cell by being deeply infolded into the cell at frequent intervals (Fig. 9). These infoldings extend apically for about four-fifths of the height of the cell (Figs. 7, 8), sometimes branching and anastomosing in their course, forming an intricately interconnected labyrinth. These infolded membranes are each about 80 Å thick and are associated in pairs which are not less than 100 Å apart. In some instances the membrane pairs are found to be widely separated and the material between them is of low density (Fig. 10).

(iii) *Mitochondria.* Mitochondria occur densely in the basal two-thirds of the cell, rapidly becoming sparse as the furthermost apical extension of the basal lamellae is approached (Figs. 8, 11). In the apical quarter or fifth of the cell there are only very occasional mitochondria. Mitochondria occupying the basal half of the cell are frequently elongated along the basal–apical cell axis and may be in intimate contact over a considerable portion of their surface with the infolded basal lamellae (Fig. 9). Their structure is similar to that of mitochondria in the lipophilic cells, and, most often, the cristae are roughly at right angles to the long axis of the mitochondrion. On the other hand, the appearance of the septa as a series of roughly concentric rings, as in Fig. 11, can probably be explained by assuming that here the cristae follow the curve of a surface of the mitochondrion and have been sectioned in a plane parallel to the tangent to this surface. Similar concentric rings have been observed in sections of mitochondria in lipophilic cells.

(iv) *Cytoplasm.* The cytoplasm is very much less dense than that of the lipophilic cells, and contains numerous small, dense granules (110–150 Å diameter), presumed to be ribonucleoprotein, scattered singly or in groups throughout it.

Membranes of the endoplasmic reticulum are sparsely distributed and are seldom seen in the apical region of the cell (Figs. 1, 11). There are fewer vesicles bounded by smooth membranes than in the lipophilic cells.

(c) *Basement membrane and connective tissue*

The basement membrane (Figs. 9, 10, 13, 14) is an apparently structureless layer about 400–600 Å wide. It has a relatively low opacity, distinctly lower, for example, than the basal membrane which, for the most part, it closely invests. Separated from the basement membrane, but generally investing some of the gut musculature and the finer tracheae supplying the gut, is a connective tissue sheath (Fig. 14). This is structureless, of low opacity and commonly almost twice as thick (500–800 Å wide) as the basement membrane which it closely resembles and to which it is connected at irregular intervals by strands of similar structureless material (Fig. 14). Where both longitudinal and circular gut musculature are present, these are separated by an additional layer of connective tissue.

(d) *Peritrophic membrane*

This membrane is uniform in any one larva and, in nearly fully-fed third-instar larvae, is about 0·8 μ thick. It appears to be structureless and of moderate opacity (Figs. 5, 8).

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DISCUSSION

Using the light microscope, WATERHOUSE and STAY (1955) described the lipophilic cells of *Lucilia* mid midgut zone II as characterized by a distinct striated border, a centrally or basally located nucleus, and a cytoplasm which stained uniformly with haematoxylin. Mitochondria, with which phospholipid appeared to be associated, were present throughout the cytoplasm, but appeared to be more abundant apically. Lipoid spheres were numerous and glycogen granules were distributed throughout, with a tendency to be more abundant in the apical half of the cell. On the other hand, the cuprophilic cells lacked a distinct striated border, the nucleus was apical, and the apical cytoplasm stained more heavily with haematoxylin than the basal cytoplasm. The apical half of the cell also contained more copper and iron and more dehydrogenase and cytochrome oxidase activity than the basal half. By contrast, mitochondria with associated phospholipid were concentrated at the base of the cells and appeared as refractile granules in stained preparations.

It is clear, therefore, that the present observations provide detailed confirmation of many of the features already described. However, it is notable that, in electron micrographs, glycogen was not recognized in the lipophilic cells and neither iron nor copper in the cuprophilic cells. Furthermore, as pointed out by WATERHOUSE and STAY (1955), it is unusual that dehydrogenase activity (as indicated by formazan granules) occurred in the apical half, whereas mitochondria were mainly located in the basal half of the cuprophilic cells. Either dehydrogenase activity is not predominantly associated with mitochondria, as in most other tissues, or the apical concentration of formazan is an artefact.

BEAMS and ANDERSON (1957) have published the only paper dealing with the fine structure of the insect midgut and so little is known of other insect tissues that it is not possible to decide whether certain features described here for *Lucilia* are actually as unusual as they now seem. Certainly the lumen borders of both lipophilic and cuprophilic cells differ strikingly from the usual picture of a striated border. The striated border consists of well-defined, individual microvilli in the larva of the moth *Malacosoma* (BEAMS and ANDERSON, 1957), in the grasshopper *Melanoplus* (BEAMS and ANDERSON, 1957), in four other cell types in the *Lucilia* midgut, in the cockroach *Periplaneta*, and in the larva of the clothes moth *Tineola* (unpublished observations). It has been inferred that the microvilli are fingerlike wherever the peritrophic membrane has a network pattern (WATERHOUSE, 1957). Microvilli also form the striated border of the malpighian tubules of *Melanoplus* (BEAMS *et al.*, 1955) and of the vertebrate intestinal epithelium (ZETTERQVIST, 1956). On the other hand, a lamellar striated border, somewhat similar to that of the *Lucilia* lipophilic cells, has been reported in the goblet cells of the nephron of the toad *Xenopus* (BARGMANN *et al.*, 1955). We also have evidence of a lamellar border in the mid midgut of *Drosophila* larvae and, in *Lucilia* larvae, in some cells of one region of the anterior midgut and in all cells in zones I and III of the mid midgut. The very low border of the *Lucilia* cuprophilic cell with its sparse and irregular microvilli would also appear to be somewhat unusual, although some difference might, perhaps, be expected in view of the probable origin of cuprophilic cells from pole cells (POULSON and WATERHOUSE, 1958). It is interesting to note, however, that the surface epithelial and chief cells of the mouse stomach have very short microvilli (DALTON, 1951) reminiscent of those of the cuprophilic cells in *Lucilia*.

The microvilli forming the striated border of the *Malacosoma* midgut are long compared with the lamellae of *Lucilia* and are thrown into a series of twists about half-way along their length. They appear to have their origin rather deeply within the apical region of the cell. The microvilli of the *Melanoplus* midgut are much larger and uncoiled. Situated between them is a spiral filament of unknown homology (BEAMS and ANDERSON, 1957). The microvilli in the *Melanoplus* malpighian tubules are fairly similar in diameter to those of the midgut, but differ markedly in the occurrence within them of the filamentous mitochondria (BEAMS *et al.*, 1955). Mitochondria also occur in the microvilli at the base of the goblet cell cavity of several lepidopterous larvae (XEROS, personal communication).

Membranes, similar to those formed in *Lucilia* as a result of the extensive infolding of the basal membrane of the cuprophilic cell, occur at least in mid midgut zone V and the posterior midgut and hindgut of *Lucilia* (unpublished observations) and in both the midgut and malpighian tubules of *Melanoplus*, but not in the midgut of *Malacosoma* (BEAMS and ANDERSON, 1957; BEAMS *et al.*, 1955). They also occur in the malpighian tubules of the waxmoth *Galleria* (BRADFIELD, 1953) and the cricket *Gryllus* (BERKALOFF, 1958, 1959). Similar infoldings have been reported in the nephron tubule of the crayfish (BEAMS *et al.*,

1956), the coxal gland of the scorpion (RASMONT *et al.*, 1958), and their occurrence in various vertebrate epithelia involved in water transport has been dealt with by PEASE (1956). Such epithelia include, for mouse or rat, the proximal tubule of the kidney, the submaxillary gland, the choroid plexus of the brain, and the ciliary body of the eye. All of our evidence in *Lucilia* suggests that the structure of the infoldings is a complicated three-dimensional labyrinth reminiscent of that described for the proximal convoluted tubule of mammalian kidney (PEASE, 1955; RUSKA *et al.*, 1957). There is, however, no evidence for any appreciable interlocking of adjacent cells as suggested for mammalian kidney (CLARK, 1957; RHODIN, 1958), and the form of the infoldings adjacent to the lipophilic cell is similar to any other basal region of the cell (Fig. 13). In a few cuprophilic cells the spaces between the membranes are, by comparison, very large (Fig. 10), as in the rat kidney (RUSKA *et al.*, 1957). This may be due to their having been distended by an aqueous solution, which, presumably, is involved in the passage of materials through the cell. Infolding as a modification for greatly increasing the surface area of the basal region of a cell undoubtedly occurs very widely in certain specialized tissues. There is little doubt that the great increases in cell surface observed in these two cells—in the lipophilic cell at the lumen surface and in the cuprophilic cell at the haemolymph surface—both serve, in different ways, to enhance the exchange of materials between gut lumen and haemolymph.

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Membranes of the endoplasmic reticulum are not a very striking feature of the lipophilic or cuprophilic cells of *Lucilia*. The Golgi complex is also not highly organized as it is in many other animal tissues. In the lipophilic cells there are groups of membrane-bounded vacuoles which, in the absence of more highly organized structures, are possibly Golgi elements. However, even these structures occur so infrequently in the cuprophilic cells that it is not possible on our evidence to designate therein any organelles as possible Golgi elements.

Connective tissue in insects is generally inconspicuous and has been little studied. Nevertheless, it is widespread in the form of basement membranes, tissue sheaths, and supporting strands. In some situations (BACCETTI, 1957; EDWARDS *et al.*, 1958a, b; GRAY, 1959; WATERHOUSE, 1950) there is evidence of a layered, banded, or filamentous structure. The present study demonstrates the presence of two or more similar and interconnected concentric sheaths enclosing the midgut. One of these sheaths of supporting tissue is the basement membrane of the gut and the other encloses the finer branches of the tracheal supply to the gut, together with some of the gut musculature.

It is abundantly clear that further study of the insect midgut with the electron microscope will throw a great deal of light on the structure and, together with histochemical tests, on the probable function of the various highly differentiated cells which form it.

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HISTOLOGISCHE UND EXPERIMENTELLE UNTERSUCHUNGEN ÜBER DIE AUSLÖSUNG DER METAMORPHOSE BEI *LEUCOPHAEA MADERAE* (ORTHOPTERA)*

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Abstract—Metamorphosis in insects is brought about by a relative decrease or lack of juvenile hormone secretion during the last larval instar. The relative inactivity of the corpora allata in the last instar can be due to a negative allometric growth of these glands or to an inactivation which is brought about by a physiological activity of other organs. These possibilities have been investigated in the roach *Leucophaea* by means of histological studies of the growth of the c. allata during larval development and by experimental procedures which resulted in the activation of the c. allata during the last larval instar.

The volume of the gland and the nuclear density are equally important for judging c. allata-activity. We have therefore used a combination of both values, the 'activity volume' (difference between the volume of the gland and the minimal volume of a gland with an equal number of nuclei). The activity volume first decreases, but increases rapidly after the third moult. During the last larval instar it drops to zero.

After killing a certain number of pericardial cells or after sectioning the c. allatum nerves soon after the last larval moult, the activity volume of the c. allata fails to drop while mitotic activity is reduced. The c. allata retain their activity and this results in supernumerary moults. It is evident therefore that the c. allata are being inactivated in normal development by nervous stimuli from the protocerebrum in the same way as in adult females. The slightly negative allometric growth of the c. allata does not account for the functional insufficiency in the last instar.

In spite of the fact that the destruction of a certain number of pericardial cells at the beginning of the last larval instar leads to an activation of the c. allata and to supernumerary larval moults, the histological and experimental evidence is against the idea which we have put forward earlier, that the pericardial cells have a function in inducing metamorphosis by inhibiting the c. allata. It is more probable that the effect observed after our operation is brought about by the injury.

EINLEITUNG

Die Metamorphose der Insekten kommt dadurch zustande, dass die Corpora allata im letzten Larvenstadium die Sekretion von Juvenilhormon einstellen oder dieses nur in ungenügender Menge produzieren. Ohne Juvenilhormon bewirkt das Hormon der Prothorakaldrüse (Ecdyson) eine imaginale oder pupale Differenzierung. Die Frage, ob die C. allata gehemmt werden (Wigglesworth, 1954)

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oder ob die Sekretion infolge eines negativ allometrischen Wachstums der C. allata im Verhältnis zum Körpergewicht dauernd zurückgeht, bis schliesslich die ausgeschüttete Juvenilhormonmenge unterhalb einer bestimmten Schwelle bleibt (KAISER, 1949; NOVÁK, 1959) ist noch umstritten. Ein Zufallsergebnis, die Erzeugung von Extralarven infolge der ungewollten Zerstörung von Pericardialzellen bei Kastrationsversuchen und anschliessend ausgeführte Ausschaltversuche, führte zur Vermutung, dass die Pericardialzellen im letzten Larvenstadium durch Abgabe eines Stoffes die C. allata hemmen und dadurch die Metamorphose auslösen (ENGELMANN und LÜSCHER, 1956a). Diese Vermutung stützte sich ausser auf die Ausschaltversuche auf die histologischen Untersuchungen von SCHWINCK (1951), die bei *Panorpa* eine starke Vermehrung der Pericardialzellen im Laufe der Larvenentwicklung festgestellt hatte.

Wir haben uns nun zur Aufgabe gestellt, die Funktion der Pericardialzellen durch Transplantations- und Ausschaltversuche, sowie durch histologische Untersuchungen an *Leucophaea* weiter zu analysieren. Um die postulierte Wirkung auf die C. allata zu beweisen, sowie um die Frage der Hemmung derselben und die Folgen des allometrischen Wachstums abzuklären, waren auch histologische Untersuchungen der C. allata unter normalen und experimentellen Bedingungen erforderlich. Dabei haben wir den Versuch unternommen, aus dem Drüsenvolumen, der Zellenzahl und dem Zellvolumen eine Grösse abzuleiten, die gute Indizien über die Aktivität des Organs geben kann.

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MATERIAL UND TECHNIK

Die für unsere Untersuchungen verwendeten Tiere entstammten der Zucht des Zoologischen Instituts Bern, die ursprünglich aus der Zucht von Frau Dr. B. SCHARRER, New York, hervorgegangen ist. Die Schaben wurden bei 26°C und bei 85–90 Prozent relativer Luftfeuchtigkeit gezüchtet und mit Hundkuchen, gelben Rüben und Äpfeln gefüttert. Unter diesen Zuchtbedingungen machen die Schaben in weitaus den meisten Fällen 8 Larvenstadien durch.

Zur Bestimmung des Volumens der C. allata wurden diese in Schnittserien zerlegt. Jeder einzelne Schnitt wurde gezeichnet und planimetriert. Die Kernzählungen erfolgten nach der von ENGELMANN (1957) beschriebenen Methode. Die Pericardialzellen wurden durch Injektion von Trypanblau (1-prozentige Lösung in Ringer) ins lebende Tier sichtbar gemacht (PALM, 1952). Bei Fixierung mit Susa blieb der in den Zellen gespeicherte Farbstoff erhalten. Die Auszählung der Septum-Pericardialzellen im dorsalen Diaphragma erfolgte anhand von Totalpräparaten. Diese wurden zur Bestimmung des Volumens der Pericardialzellen später in Schnittserien zerlegt.

Die Operationen wurden unter CO_2-Narkose durchgeführt. Zur Ausschaltung von Pericardialzellen und Gehirnpartien diente das Hochfrequenz-Mikrochirurgiegerät 'Dermopytor' der Firma Sanitas, Berlin. Zur Koagulation der Pericardialzellen wurde die Nadel von hinten beiderseits des Herzschlauches in jedes Segment einzeln eingestochen.

HISTOLOGISCHE UNTERSUCHUNGEN

(a) *Die Corpora allata*

Nachdem für adulte Weibchen von *Leucophaea* gezeigt werden konnte, dass Volumen und Kerndichte der C. allata mit der Aktivität schwanken, war anzunehmen, dass diese Grössen auch während der Larvenentwicklung Indizien über die Aktivität der Drüsen liefern können, umsomehr als nach unseren Messungen die Grösse der Zellkerne während der ganzen Larvenentwicklung gleich bleibt, also keine Polyploidisierung erfolgt. Analog zu den Verhältnissen beim adulten Weibchen dürften deshalb auch bei der Larve eine Volumenzunahme und eine Verringerung der Kerndichte (Kernzahl pro 1 mm³) einer Zunahme der Aktivität der Drüsen entsprechen; die Kerndichte ist dem durchschnittlichen Zellvolumen*

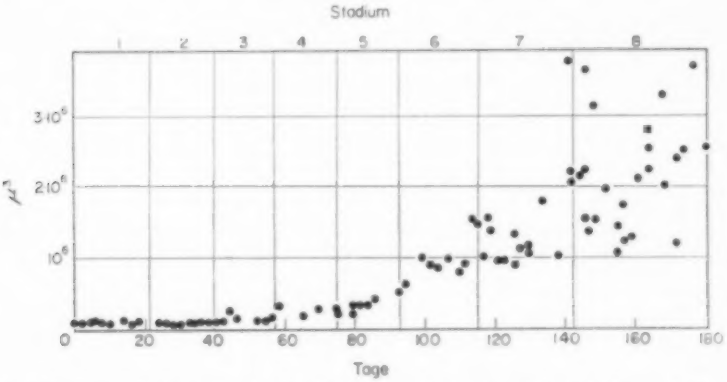

ABB. 1. Volumenveränderungen der C. allata im Laufe der Larvenentwicklung. Jeder Punkt entspricht dem Durchschnittswert aus beiden Drüsen eines Tieres.

umgekehrt proportional. Bei völliger Inaktivität kurz vor der Metamorphose beträgt das durchschnittliche Zellvolumen 1000 μ^3, im aktiven Zustand aber bedeutend mehr. Da das durchschnittliche Kernvolumen gleich bleibt, betrifft die Volumen-vergrösserung nur das Cytoplasma. Die Volumendifferenz dürfte der Aktivität entsprechen und ist wohl das beste Mass, das uns aus der histologischen Untersuchung zur Beurteilung der Aktivität der Drüse zur Verfügung steht; wir bezeichnen diese Volumendifferenz als 'Aktivitätsvolumen'. Das von uns berechnete Aktivitätsvolumen einer Drüse entspricht der Differenz zwischen dem Volumen und dem aus der absoluten Kernzahl für die gleiche Drüse bei Inaktivität berechneten Volumen (unter Zugrundelegung des Zellvolumens von 1000 μ^3).

Die Ergebnisse unserer Untersuchungen sind in Abb. 1–6 dargestellt. Das C. allata-Volumen (Abb. 1) zeigt ein ganz schwaches Wachstum während der ersten drei Stadien, dann eine allmählich stärker werdende Vergrösserung bis zum

* Unter Zellvolumen verstehen wir das durchschnittliche Drüsenvolumen pro Zellkern. Es entspricht dem tatsächlichen Zellvolumen nur unter der Voraussetzung, dass es sich um einkernige Zellen handelt, was nicht erwiesen ist.

Ende des siebenten Stadiums. Während des letzten Larvenstadiums geht das Volumen anfangs stark zurück, steigt aber etwa 20 Tage vor der Adulthäutung wieder ziemlich stark an. Vom 7. Stadium an ist das Volumen stets zur Zeit der Häutung am grössten. Dies entspricht den Erwartungen insofern nicht, als die C. allata eigentlich vorher, zur Zeit der Ausschüttung des Häutungshormons durch die Prothorakaldrüsen, aktiv sein müssten, damit imaginale Differenzierungen verhindert werden.

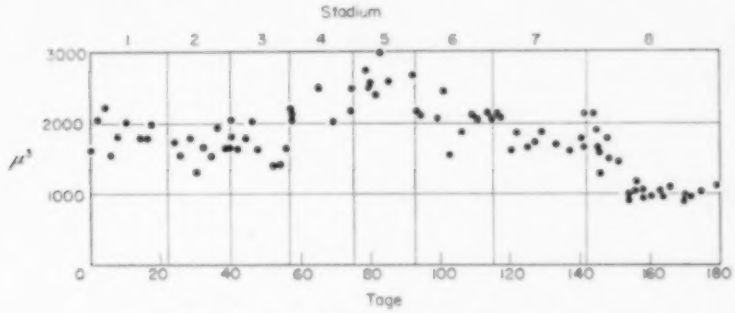

ABB. 2. Das durchschnittliche Zellvolumen in den C. allata.

Das durchschnittliche Zellvolumen, das der Zelldichte umgekehrt proportional ist (Abb. 2) nimmt in den ersten Stadien etwas ab, steigt auf ein Maximum im 5. Stadium sinkt dann allmählich wieder etwas, um zu Beginn des letzten

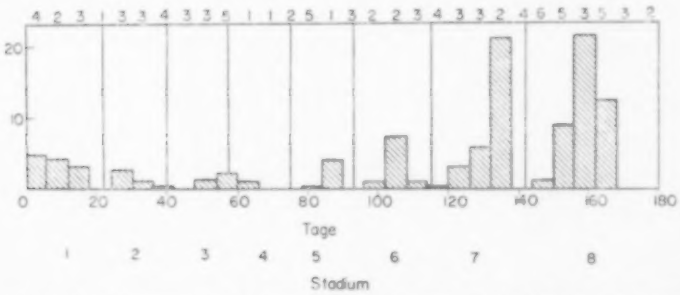

ABB. 3. Zahl der festgestellten Mitosen in den C. allata. Die Höhe der Säulen entspricht dem Durchschnitt der pro Tier gefundenen Mitosen. Die Zahl über den Säulen gibt die Zahl der Tiere an, die zur Berechnung des Durchschnittswertes zur Verfügung standen.

Stadiums stark abzusinken. Es erreicht hier wahrscheinlich im Zustand völliger Inaktivität sein Minimum. Schwankungen innerhalb der Stadien sind ausser im letzten Stadium kaum zu bemerken, mit Ausnahme leichter Vergrösserungen zur Zeit der Häutung am Ende des 6. und 7. Stadiums.

Abb. 3 zeigt die durchschnittlich in den untersuchten Schnittpräparaten festgestellten Mitosen. Relativ viele Zellteilungen wurden im 1. Stadium festgestellt, obschon die absolute Zellzahl im 2. Stadium kaum höher ist (Abb. 4). Vermehrte Mitosen treten dann nach der Mitte der Stadien 5–7 auf, dadurch

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kommt es zu einer starken Volumenvergrösserung trotz abnehmendem Zellvolumen am Ende dieser Stadien. Im letzten Larvenstadium treten viele Mitosen in der ersten Hälfte des Häutungsintervalles auf.

Aus Zellvolumen und Drüsenvolumen lässt sich die absolute Zellenzahl (Abb. 4) berechnen. Während der ersten vier Stadien nimmt die Zahl der Zellen nur schwach, später progressiv immer mehr zu. Es ist bemerkenswert, dass die

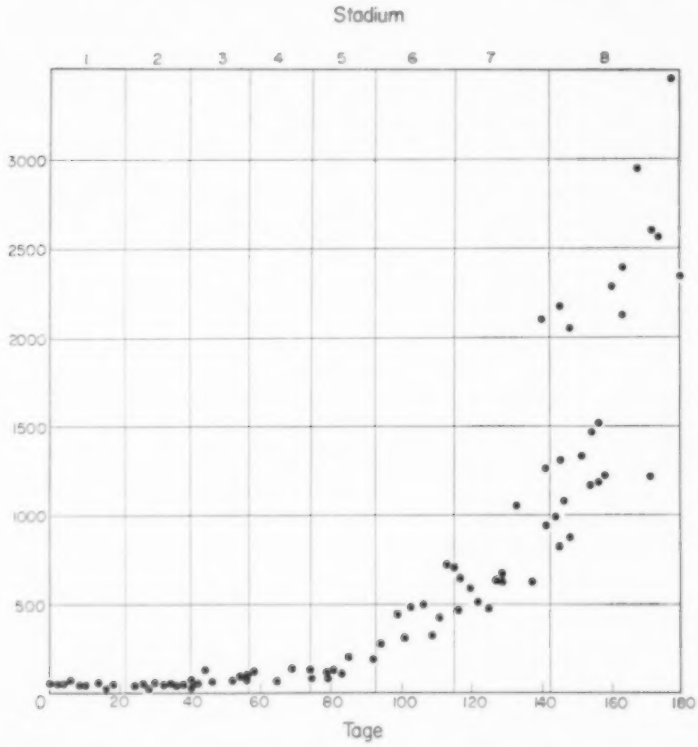

ABB. 4. Zahl der Zellen pro Corpus allatum. Jeder Punkt entspricht dem aus den beiden Drüsen eines Tieres ermittelten Durchschnitt.

starke Zunahme dann beginnt, wenn das Zellvolumen kleiner wird, sodass das Drüsenvolumen durch diese Umstellung nicht beeinflusst wird (Abb. 6). Eine starke Zunahme der Zellen erfolgt ausser im letzten Stadium nur in der zweiten Hälfte jedes Stadiums (Abb. 4). Sie beginnt in der Zeit, zu welcher wir etwa die grösste Aktivität erwarten dürften, und zu welcher wohl auch die Prothorakaldrüsen aktiv sind.

Das Aktivitätsvolumen (Abb. 5) sinkt im 2. Stadium auf ein Minimum und nimmt vom 4. Stadium an sehr stark zu. Im 8. Stadium sinkt es etwa 25 Tage vor der Adulthäutung auf Null. Merkwürdigerweise lässt sich innerhalb nahezu

jeden Stadiums ein Maximum zum Zeitpunkt der Häutung feststellen. Dies könnte darauf schliessen lassen, dass die höchste Aktivität nicht mit dem Maximum an Cytoplasma, sondern mit der grössten Volumenzunahme zusammenfällt, wobei die Volumenzunahme eventuell eine sekundäre Erscheinung sein könnte.

Die logarithmische Darstellung, die nur die zum Zeitpunkt der Häutungen gefundenen Werte berücksichtigt (Abb. 6) gestattet es, die Zunahme des Drüsenvolumens, des Aktivitätsvolumens und der Zellzahl mit dem Wachstum des

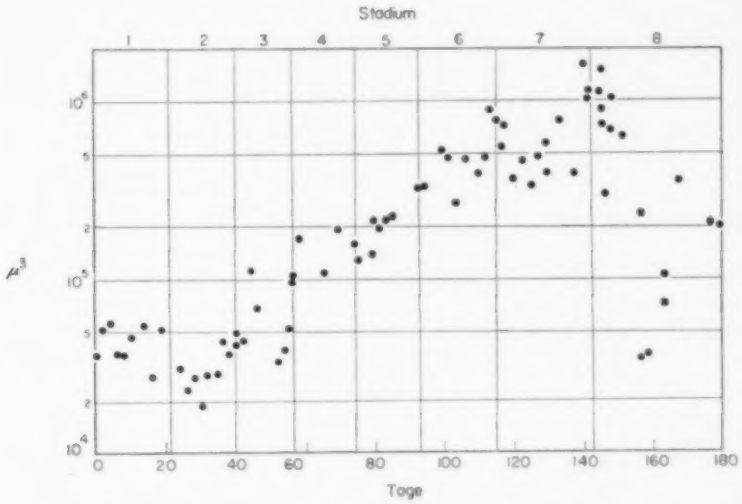

ABB. 5. Das Aktivitätsvolumen der C. allata. Jeder Punkt entspricht dem Durchschnitt aus den zwei Drüsen eines Tieres. Im letzten Larvenstadium (8) sinkt die Kurve am 152. Tag der Entwicklung auf Null. Dies ist aus der Abbildung nicht ersichtlich, da 7 Punkte unter Null liegen und deshalb logarithmisch nicht dargestellt werden können.

Insekts (Körpergewicht) in Beziehung zu setzen. Das Gewicht nimmt von Stadium zu Stadium um den Faktor 1,88 zu. Es wird also in jedem Stadium nahezu verdoppelt. Das Wachstum der C. allata bleibt anfänglich stark hinter demjenigen des ganzen Tieres zurück. Vom 4. bis zum 7. Stadium jedoch haben die Drüsen dieselbe oder eine noch höhere Wachstumsrate. Erst im letzten Stadium bleibt das Wachstum wieder stark zurück. Die absolute Zellzahl nimmt während der ersten vier Stadien ziemlich konstant um den Faktor 1,29 zu während der letzten vier Stadien um den Faktor 2,19. In dieser Zeit teilt sich also jede Zelle mindestens einmal in jedem Stadium, einzelne Zellen sogar zweimal. Im fünften Stadium erfolgt eine völlige Umstellung der Zellteilungsrate, die mit der beginnenden Abnahme des Zellvolumens zusammenfällt und deren Ursache nicht ersichtlich ist. In der Normalentwicklung ist die Zellteilungsrate völlig unabhängig von der Aktivität der Drüsen. Sie ist bei Inaktivität im letzten Stadium gleich gross wie bei Aktivität im 5., 6. und 7. Stadium. Das Aktivitätsvolumen nimmt im Verhältnis zum Körpergewicht vom 4. bis 6. Stadium stark

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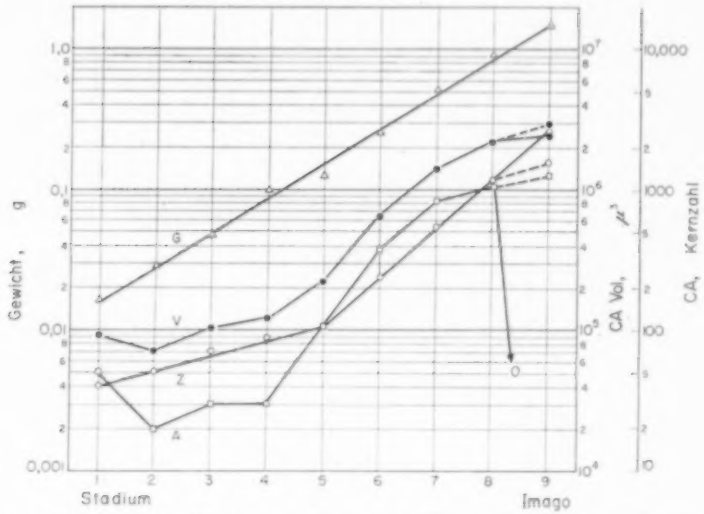

ABB. 6. Vergleich des Wachstums des ganzen Tieres mit demjenigen der C. allata. G = Körpergewicht. V = Volumen, Z = Zellzahl, und A = Aktivitätsvolumen der C. allata. Die eingezeichneten Punkte stellen Durchschnittswerte von mehreren Tieren zur Zeit des Schlüpfens aus dem Ei und der Häutung dar. Die gestrichelten Linien vor der Imaginalhäutung entsprechen den Veränderungen vor einer experimentell ausgelösten Extralarvenhäutung.

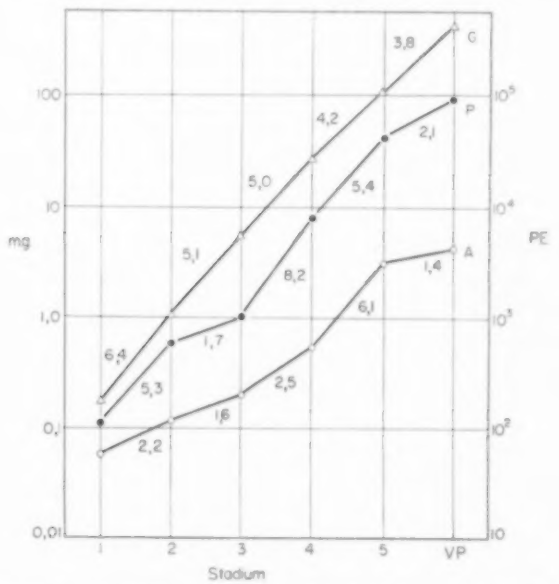

ABB. 7. Wachstum von Körpergewicht, Prothorakaldrüsen und C. allata von *Pieris*, logarithmisch dargestellt nach den Angaben von KAISER (1949). G = Körpergewicht in mg. P = Volumen der Prothorakaldrüsen, A = Volumen der C. allata in Planimeter-Einheiten (PE). VP = Vorpuppe. Die Zahlen neben den Kurven geben die Wachstumsfaktoren an.

zu. Es ist also anzunehmen, dass in dieser Zeit der Hormonspiegel im Blut von Stadium zu Stadium höher wird. Relativ am höchsten dürfte er im 6. und 7. Stadium sein. Im letzten Stadium sinkt das Aktivitätsvolumen auf Null.

Wir glauben, aus unseren histologischen Untersuchungen schliessen zu können, dass im letzten Entwicklungsstadium eine Inaktivierung der C. allata eintritt, und dass dadurch die Metamorphose ausgelöst wird. Es ist also bei *Leucophaea* nicht so, wie dies KAISER (1949) für *Pieris* vermutet hat, dass nämlich die relative Grösse der C. allata und damit die erzeugte relative Hormonmenge im Laufe der Larvenentwicklung dauernd abnimmt, sodass schliesslich das produzierte Hormon nicht mehr ausreicht, um die imaginale bezw. pupale Differenzierung zu verhindern. Wenn KAISER's Messergebnisse logarithmisch dargestellt werden (Abb. 7), so zeigt sich auch für *Pieris*, dass die C. allata nach einem allerdings nur schwachen Wachstum in den ersten Stadien während des 4. Stadiums relativ stärker wachsen als die Prothorakaldrüsen und als das ganze Insekt. Es kommt auch hier im letzten Stadium zu einer plötzlichen Verlangsamung des Wachstums der C. allata, was u.E. auf eine Inaktivierung derselben schliessen lässt.

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(b) *Die Pericardialzellen*

Nach den Angaben von SCHWINCK, 1951, nehmen die Pericardialzellen von *Panorpa* in den letzten Stadien ganz besonders stark an Zahl und Volumen zu. Auf Grund dieser Angaben und unserer Ausschaltversuche (ENGELMANN und LÜSCHER, 1956) haben wir den Pericardialzellen eine wichtige Rolle bei der Inaktivierung der C. allata im letzten Larvenstadium zugeschrieben. Es war aber noch zu prüfen, ob eine entsprechende Vermehrung der Pericardialzellen auch bei *Leucophaea* gerade im letzten Stadium auftritt.

Die Anordnung der Pericardialzellen im Abdomen von *Leucophaea* ist in Abb. 8 dargestellt. Es sind grosse, in der Regel zweikernige Zellen, die aus der Haemolymphe injizierte Farbstoffe wie Ammoniakkarmin und Trypanblau aufnehmen und speichern. Nach ihrer Lage kann man zwei Typen unterscheiden: freie Pericardialzellen, die in segmentalen Gruppen beidseitig des Herzschlauches im Pericardialsinus liegen, den bekannten Pericardialzellen der andern Insekten entsprechend, und Septumpericardialzellen (Abb. 9), die im ganzen gefensterten Pericardialseptum zerstreut liegen. Diese entsprechen in ihrer Erscheinung und in ihrer Speicherungsfähigkeit für Farbstoffe durchaus den übrigen Pericardialzellen. Vergleichbare Septumzellen wurden auch schon bei Larven von *Apis* (NELSON, 1915) und bei *Nepa* (HAMILTON, 1931) festgestellt; Septumzellen mit phagocytären Eigenschaften bei Thysanuren (PHILIPTSCHENKO, 1907; BRUNTZ, 1908).

Volumenmessungen an Pericardialzellen sind, besonders in den letzten Stadien, sehr zeitraubend. Wir haben uns deshalb darauf beschränkt, das Volumen der freien Zellen und die Zahl der Septumzellen in jedem Entwicklungsstadium nur im 5. Abdominalsegment zu bestimmen, in der Annahme, dass die Zell- und

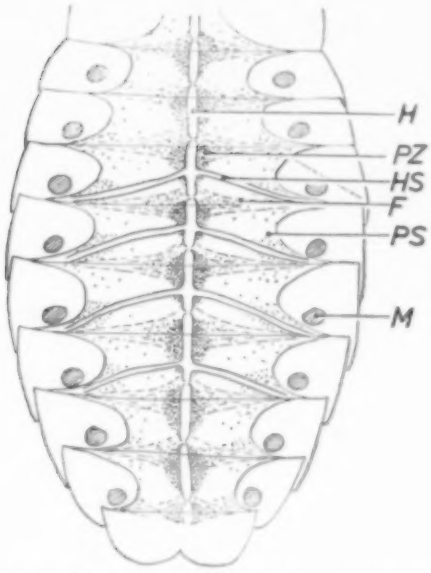

ABB. 8. Anordnung der Pericardialzellen im Abdomen von *Leucophaea*. H = Herz, HS = Seitenäste des Herzschlauchs, F = Herzflügelmuskeln, M = Dorsoventralmuskeln, PS = Septumpericardialzellen, PZ = 'freie' Pericardialzellen.

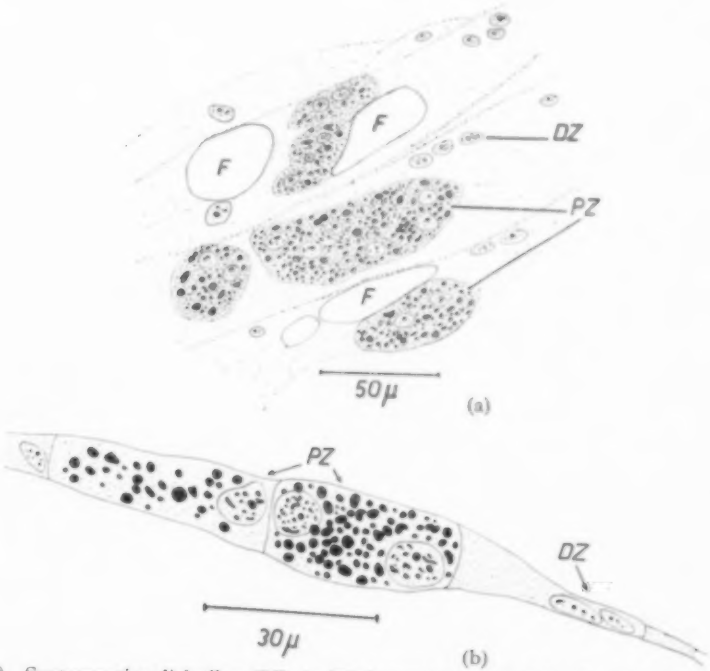

ABB. 9. Septumpericardialzellen (PZ) in Flächenansicht (a) und im Querschnitt (b). DZ = normale Diaphragmazellen, PZ = Pericardialzellen, F = Fenster im Septum.

Volumenzunahme in den anderen Segmenten vergleichbar ist. In Abb. 10 sind die Messergebnisse logarithmisch dargestellt und mit der Kurve für das Körpergewicht in Beziehung gesetzt. Die Kurven für die Zahl der Septumzellen und das Volumen der freien Zellen laufen ungefähr parallel. In den ersten Stadien

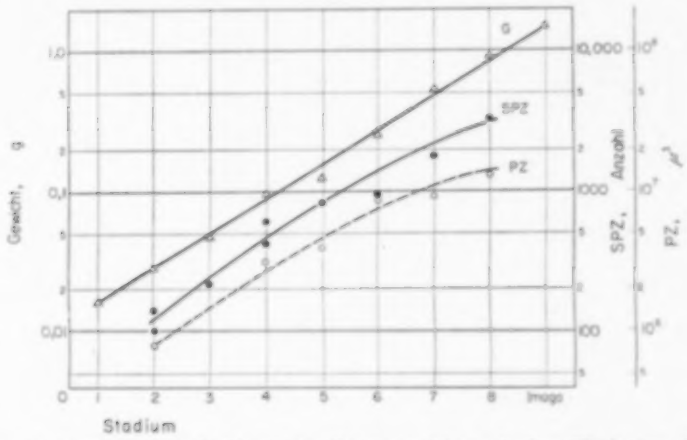

Abb. 10. Vergleich der Zunahme des Körpergewichts mit dem Wachstum der Pericardialzellen im Laufe der Larvenentwicklung von *Leucophaea*. Die Punkte der Pericardialzellenkurve sind die für das 5. Abdominalsegment gefundenen Werte, unmittelbar nach dem Schlüpfen oder nach der Häutung. G = Körpergewicht, SPZ = Anzahl Septumpericardialzellen, PZ = Volumen der 'freien' Pericardialzellen.

ist die Zunahme etwas stärker als diejenige des Körpergewichts, in den letzten Stadien geht jedoch das Pericardialzellenwachstum relativ zum Gewichtswachstum des Insekts eher etwas zurück. Aus dem Wachstum der Pericardialzellen ergibt sich deshalb keinerlei Stütze für unsere Annahme einer relativen Zunahme eines von ihnen produzierten Faktors, der für die Inaktivierung der C. allata im letzten Stadium verantwortlich wäre.

EXPERIMENTELLE UNTERSUCHUNGEN

(a) *Ausschaltung von Pericardialzellen*

Wie schon kurz mitgeteilt wurde (ENGELMANN und LÜSCHER, 1956) treten nach Ausschaltung von Pericardialzellen in vielen Fällen 1–2 extralarvale Stadien auf. Es kommt zur Entstehung von Extralarven (Abb. 11). Wir haben nun versucht, durch weitere Ausschaltversuche mittels Elektrokoagulation die Bedingungen zu präzisieren, unter denen es zu extralarvalen Häutungen kommt. Die Ergebnisse dieser Versuche sind in Tabelle 1 zusammengestellt. Es geht daraus hervor, dass sich besonders in den ersten sechs Tagen nach der letzten Larvenhäutung extralarvale Häutungen erzwingen lassen (später nur noch in einem kleinen Prozentsatz), und dass die Zahl der ausgeschalteten Zellen von grosser Bedeutung ist. Die Operationen waren nur erfolgreich, wenn ausser dem Herzschlauch und den

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anliegenden freien Pericardialzellen auch die seitlichen Septumzellen abgetötet wurden. 29 Larven des letzten Stadiums, deren Herzschlauch mit den angrenzenden freien Pericardialzellen vor dem 6. Tag auf einer Länge von 5–6 Segmenten

TABELLE 1—AUSSCHALTUNG VON PERICARDIALZELLEN BEI LARVEN DES LETZTEN STADIUMS

Operation Tage nach Häutung	Operierte Segmente	Ergebnis	
		Imagines	Extralarven
1–6	2–3½	14	10 (42%)
	4–6	3	16 (84%)
7–12	1–3½	14	0
	4–6	8	2 (20%)
13–30	2–6	55	0

ausgeschaltet wurde, machten alle eine Adulthäutung durch. Auch die Ausschaltung der Septumzellen allein führte nicht zu extralarvalen Häutungen. Auffallenderweise entstanden nie Zwischenformen zwischen Larve und Imago. Es handelt sich also um eine 'Entweder-Oder'-Reaktion.

Von 28 durch Ausschaltung von Pericardialzellen erzielten Extralarven wurden 9 weiterbeobachtet. Davon häuteten sich 7 zu Adulttieren, während 2 eine zweite extralarvale Häutung durchmachten (Abb. 11) und erst nach der dritten Häutung nach der Operation adult wurden.

Bei der Häutung zu Extralarven erfolgt ein weiteres Wachstum, sodass die resultierenden Adulttiere viel grösser sind als die normalen Imagines. Abb. 11 zeigt einen Grössenvergleich einer normalen Larve des letzten Stadiums mit Extralarven nach einer und nach zwei Häutungen nach der Operation.

(b) *Die Wirkung der Ausschaltung der Pericardialzellen auf die C. allata*

Wenn die Ausschaltung eines quantitativen Minimums von Pericardialzellen zu Beginn des letzten Larvenstadiums zu einer Verhinderung der Metamorphose führt, so ist anzunehmen, dass die C. allata indirekt durch die Operation beeinflusst werden, da ja normalerweise die Metamorphose durch die Inaktivierung der C. allata eingeleitet wird. Um die Wirkung der Operation auf die C. allata zu untersuchen, wurden 28 am 5. Tag des letzten Stadiums operierte Tiere fortlaufend in Abständen von 3–4 Tagen fixiert. Als Mass für die Aktivität der C. allata wurde das durchschnittliche Zellvolumen bestimmt. Das Ergebnis ist in Abb. 12 dargestellt. Die gefundenen Werte lassen sich deutlich in zwei Kategorien sondern, eine mit einer schwächeren Aktivität (unter 1300 μ^3) und eine mit einer stärkeren (1500–2000 μ^3). Nur bei wenigen Punkten erscheint die Zuordnung zur einen oder anderen Kategorie zweifelhaft. Es darf wohl angenommen werden, dass die Larven mit stärkerer C. allata-Aktivität eine extralarvale Häutung durchgemacht hätten (die C. allata-Aktivität unmittelbar nach

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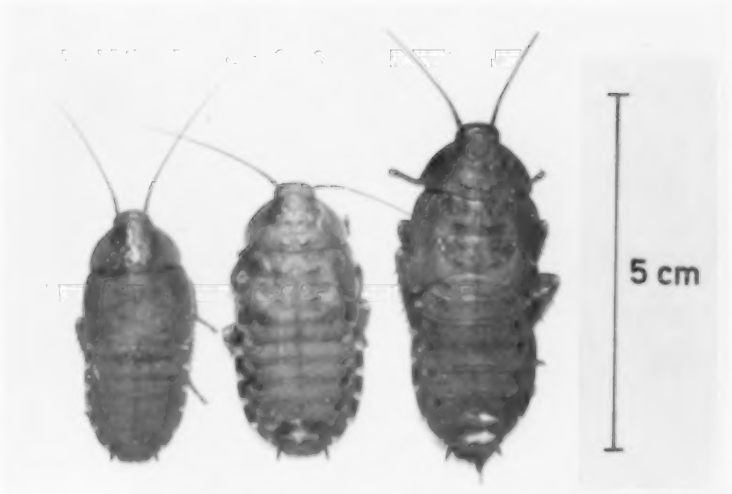

ABB. 11. Grössenvergleich einer normalen Larve des letzten Stadiums (links) mit Extralarven nach einer (Mitte) und nach zwei extralarvalen Häutungen (rechts).

einer extralarvalen Häutung (MH) ist histologisch ganz entsprechend), und dass die Larven mit schwacher Aktivität, bezw. Inaktivität der C. allata (entsprechend den Normalverhältnissen im letzten Stadium) vor einer Imaginalhäutung standen. Wie die gestrichelte Kurve in Abb. 12 zeigt, erfolgt nach der Ausschaltung der Pericardialzellen, sofern es zu extralarvalen Häutungen kommt, keine Aktivierung der C. allata, sondern die normalerweise eintretende Inaktivierung unterbleibt.

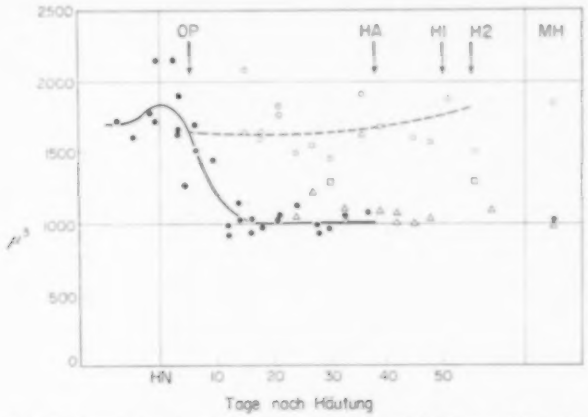

ABB. 12. Die Wirkung der Ausschaltung der Pericardialzellen auf das durchschnittliche Zellvolumen der C. allata. Schwarze Punkte = nicht operierte Kontrolltiere. Umrahmte Punkte = operierte Tiere: Kreise = voraussichtlich Extralarven. Dreiecke = voraussichtlich Imagines, Vierecke = Entwicklung ungewiss. HN = letzte Larvenhäutung, OP = Zeitpunkt der Operation, HA = durchschnittlicher Zeitpunkt der Häutung der Kontrollarven zu Imagines, $H_{1,2}$ = durchschnittlicher Zeitpunkt der Häutung operierter Larven zu Imagines (H_1) und zu Extralarven (H_2). MH = Zellvolumen in den C. allata nach der nächsten Häutung.

Während normalerweise im letzten Larvenstadium viele Mitosen in den C. allata ablaufen (Abb. 3), wurden bei den operierten Tieren mit aktiven C. allata nur wenige Zellteilungen beobachtet. Es stellt sich deshalb die Frage, ob zwischen der Mitosetätigkeit und der Aktivität eine Beziehung besteht. Zur Abklärung dieser Frage wurden bei Larven des letzten Stadiums, bei Extralarven und frisch gehäuteten Adulttieren Volumina und Kerndichte der C. allata bestimmt und daraus die absolute Kernzahl pro Drüse errechnet (Tabelle 2). Es zeigt sich, dass vor einer extralarvalen Häutung keine signifikante Zellvermehrung erfolgt. Eine Unterdrückung von Zellteilungen lässt sich mit dem *t*-Test sichern ($P < 0{,}01$). Es scheint also, dass die bei der normalen Entwicklung eintretende Inaktivierung die mitotische Aktivität in den C. allata begünstigt.

Es stellt sich nun die Frage, ob die C. allata durch die Folgen der Operation direkt oder indirekt beeinflusst werden. Bei adulten Weibchen von *Leucophaea* wird die Funktion der C. allata vom Protocerebrum (SCHARRER, 1952; ENGELMANN und LÜSCHER, 1956b) aus gesteuert. Zwei Gehirnzentren hemmen während der Trächtigkeit auf nervösem Weg die C. allata.

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Um zu prüfen, ob auch bei den Larven des letzten Stadiums die C. allata vom Protocerebrum aus gehemmt werden, wurde versucht, bei 28 Larven die C. allatum-Nerven zu durchschneiden. Eine Operation, die schon SCHARRER (1952) durchgeführt hat. Wir konnten die Resultate von SCHARRER bestätigen. In 20 Fällen gelang die Operation. 10 Larven, die erst nach der Inaktivierung der C. allata operiert wurden, häuteten sich zu Imagines, während 10 vor dem 5. Tag

TABELLE 2—DIE CORPORA ALLATA NACH DER AUSSCHALTUNG VON PERICARDIALZELLEN

Stadium nach der Häutung	Anzahl Tiere	Corpora allata		
		Volumen pro Drüse Millionen μ 3	Kernzahl pro 1 mm³ Millionen	Kernzahl pro Drüse
Kontrolle I Larve, (8. Stad.) normal	6	2,59 ± 0,39	0,55 ± 0,02	1450 ± 230
Kontrolle II Imago, normal	4	2,58 ± 0,41	0,98 ± 0,03	2530 ± 470
Versuch I Extralarve	12	2,92 ± 0,24	0,55 ± 0,02	1680 ± 120
Versuch II Imago	5	2,60 ± 0,26	1,01 ± 0,02	2610 ± 160
Versuch III Imago nach der 2. Häutung	6	2,22 ± 0,23	0,99 ± 0,01	2180 ± 230
Versuch IV Imago nach der 3. Häutung	1	2,68	0,96	2560

operierte Tiere sich zu Riesenlarven weiterhäuteten. Von diesen wurden 4 weiterbeobachtet; 3 wurden nach einer weiteren Häutung zu Imagines; das 4. Tier aber häutete sich nochmals larval. Bei allen histologisch untersuchten Extralarven waren die C. allata aktiv, bei den nach der Operation entstandenen Imagines aber inaktiv (Tabelle 3). Auch in diesem Falle war bei den experimentell erzeugten Extralarven die Zellvermehrung in den C. allata unterdrückt worden (durchschnittliche Zellzahl pro Drüse = 1720).

Das Ergebnis der Nervdurchtrennungsversuche zeigt, dass das Prinzip der nervösen Steuerung der C. allata nicht nur für die Imagines, sondern auch für die Larven des letzten Stadiums Geltung hat. Die C. allata werden durch die Ausschaltung der Pericardialzellen wahrscheinlich nicht direkt beeinflusst, sondern über die Hemmzentren im Gehirn.

Die Frage, weshalb die C. allata nach Durchtrennung der C. allatum-Nerven nicht dauernd aktiv bleiben (Tabelle 3), lässt sich noch nicht mit Sicherheit

beantworten. Die histologische Untersuchung von zwei nach einer Extralarvenhäutung entstandenen Imagines ergab, dass die Nervenverbindung in den C. cardiaca intakt war, dass also offenbar die C. allatum-Nerven regeneriert waren.

TABELLE 3—CORPORA ALLATA NACH DURCHTRENNUNG DER C. ALLATUM-NERVEN BEI LARVEN DES LETZTEN STADIUMS

Operation Tage nach der letzten Larvenhäutung	Fixiert nach	Stadium	Anzahl Tiere	Corpora allata Kernzahl pro 1 mm³ Millionen Kerne
	1. Häutung	Extralarve	5	0,51 ± 0,02
0–5	2. Häutung	Extralarve	1	0,36*
		Imago	3	0,89 ± 0,03*
15–25	1. Häutung	Imago	10	0,87 ± 0,01*

* In einigen Fällen wurde nur ein C. allatum-Nerv durchtrennt. In diesen Fällen wurde nur das C. allatum der Seite mit durchtrenntem Nerv ausgewertet.

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Das teilweise Vorhandensein von Neurosekret im caudalen Teil der C. cardiaca liess auch darauf schliessen, dass die Nerven aus der Pars intercerebralis mindestens zum Teil regeneriert waren.

(c) *Die Implantation von Pericardialzellen*

Wenn die Pericardialzellen für die Hemmung der C. allata und damit für die Auslösung der Metamorphose verantwortlich sind, wie aus den Ausschaltversuchen hervorzugehen scheint, so sollte es auch möglich sein, durch Implantation von Pericardialzellen aus Larven des letzten Stadiums in solche früherer Stadien eine vorzeitige Metamorphose auszulösen. Wir haben den Herzschlauch mit den anhängenden Pericardialzellen aus Larven des letzten Stadiums entnommen und am 1.–3. Tag nach der Häutung in Larven des 6. oder 7. Stadiums implantiert. In einigen Fällen wurden Pericardialzellen aus drei Larven des letzten Stadiums in ein Tier implantiert. Bei keiner der 14 so operierten Larven konnte eine verfrühte Metamorphose ausgelöst werden. Weiteren 11 Larven wurden Pericardialzellen zusammen mit dem Pericardialseptum aus einer, meist aber aus zwei Larven des letzten Stadiums implantiert. Auch in diesen Fällen erfolgte eine normale Larvenhäutung. Die histologische Untersuchung dieser Tiere ergab, dass die implantierten Pericardialzellen intakt waren. Sie vermochten sogar nach Injektion von Trypanblau diesen Farbstoff aufzunehmen. Sie waren also anscheinend normal funktionierend.

Eine verfrühte Metamorphose liess sich also durch Implantation von Pericardialzellen nicht erzielen. Wir müssen daraus schliessen, dass entweder die Pericardialzellen nicht den ihnen zugeschriebenen Metamorphosefaktor (ENGELMANN und LÜSCHER, 1956a) abgeben, oder dass weitere Faktoren des Larvenmilieus die Pericardialzellen erst zu dieser Funktion anregen.

DISKUSSION

(a) *Die Bedeutung der Pericardialzellen im Metamorphosegeschehen*

Die histologischen Untersuchungen haben gezeigt, dass die Pericardialzellen im Laufe der Entwicklung ziemlich gleichmässig an Zahl und Volumen zunehmen und dass die Zunahme gegen das letzte Larvenstadium hin eher etwas zurückgeht (Abb. 10). Aus der Zellvermehrung kann also nicht auf eine spezielle Aktivität im letzten Stadium geschlossen werden. Die negativen Ergebnisse der Versuche, bei Larven eine verfrühte Metamorphose auszulösen, sprechen sogar gegen eine Wirkung der Pericardialzellen bei der Auslösung der Metamorphose, wie wir sie früher postuliert haben (ENGELMANN und LÜSCHER, 1956a). Andererseits wurde der Befund bestätigt, dass nach Ausschaltung einer bestimmten, minimalen Menge von Pericardialzellen die C. allata aktiv bleiben und die Metamorphose verhindern. Zur Erklärung dieser sich widersprechenden Befunden können zwei Möglichkeiten in Betracht gezogen werden.

Unter der Annahme, dass eine Aktivität der Pericardialzellen sich nicht in einer Vermehrung oder Vergrösserung der Zellen äussert, könnten diese Zellen im letzten Stadium einen Faktor abgeben, der das Gehirn veranlasst, die C. allata zu hemmen. Da die Aktivität der C. allata deutlich beeinflusst wird, kann die Wirkung nicht in einer Elimination von Juvenilhormon bestehen, wie sie in Analogie zu der bekannten Speicherexkretionsfunktion der Pericardialzellen erwartet werden könnte. Wenn diese Hypothese zutrifft, so müssen noch weitere Faktoren im larvalen Milieu vorhanden sein, die für die Aktivierung der Pericardialzellen notwendig sind. Nur solche Faktoren könnten die Misserfolge der Implantationsversuche erklären.

Es besteht aber auch die Möglichkeit, dass die sehr starke Verletzung, die die Ausschaltung der Pericardialzellen in vier und mehr Segmenten notwendigerweise mit sich bringt, an sich eine Wirkung auf das hormonale System ausüben kann. Ausser den Pericardialzellen werden Herzschlauch, Pericardialseptum und ansehnliche Mengen Fett- und Muskelgewebe zerstört. Alle diese Gewebe werden nach der Koagulation abgebaut, allmählich resorbiert und zum Teil regeneriert. Dies bedeutet für den Organismus eine grosse Umstellung im Stoffwechsel, der offensichtlich allgemein erhöht werden muss. Aus der Literatur sind nur wenige Fälle einer Beeinflussung des endokrinen Systems durch Verletzungen bekannt. O'FARRELL *et al.* (1956) beobachteten nach wiederholter Amputation von Beinen bei *Blattella* eine Verzögerung der Häutung oder 1 bis mehrere extralarvale Häutungen. Bei *Periplaneta* können sogar nach einmaliger Amputation beider Antennen bis zu 4 extralarvale Stadien auftreten (POHLEY, 1959). Die Amputation von Beinen und Antennen ist, verglichen mit unserer Operation, nur eine geringfügige Verletzung. Bei *Leucophaea* wird die imaginale Differenzierung durch die Amputation von Beinen nicht beeinflusst (unveröffentlichte Versuche von F. ENGELMANN). Die Regeneration von Beinen erfolgt im letzten Stadium neben der imaginalen Differenzierung sowohl bei normalen als auch bei allatektomierten Larven. Dies widerspricht den Befunden von PFLUGFELDER (1939), nach denen bei *Dixippus* in Abwesenheit der C. allata keine Regeneration

erfolgen kann. Es scheint uns jedoch wahrscheinlich, dass durch eine starke Verletzung bei *Leucophaea* das erreicht wird, was bei anderen Schaben die Amputation von Gliedmassen bewirken kann: das Aktivbleiben der C. allata. Dies spricht gegen die von uns früher postulierte Theorie des Pericardialzellenfaktors, für die noch zwei mehr oder weniger unwahrscheinliche Zusatzhypothesen (spezifischer Wirkstoff der Pericardialzellen und weitere Faktoren des Larvenmilieus) erforderlich sind. Die Tatsache, dass die Grösse der Wunde für den Effekt von ausschlaggebender Bedeutung ist, spricht ebenfalls für die Verletzungshypothese. Wenn sie richtig ist, so müssen wir annehmen, dass stoffliche Faktoren, die bei der Verletzung oder beim Zerfall verletzter Zellen entstehen, direkt oder indirekt auf das Gehirn einwirken. Bei Vorhandensein in bestimmter Minimalkonzentration müssten diese Faktoren eine Lähmung der zu Beginn des letzten Larvenstadiums aktiv werdenden Hemmzentren für die C. allata bewirken.

(b) *Die Funktion der C. allata in Larvenstadien*

Wir haben versucht, die Frage der C. allata-Aktivität in den Larvenstadien durch histologische Untersuchungen abzuklären. Als Mass für die relative Aktivität wird im allgemeinen das Drüsenvolumen benützt (KAISER, 1949; NOVÁK, 1959 u.a.). Ausserdem kann die Kerndichte oder ihr reziproker Wert, das Zellvolumen, Indizien über den Aktivitätszustand der Gewebe ergeben (ENGELMANN, 1957). Unabhängig voneinander sind beide Grössen unbefriedigend, da das Drüsenvolumen nicht den Zustand der Zellen, das Zellvolumen bezw. die Kerndichte nicht das Drüsenvolumen berücksichtigt. Wir glauben, mit dem Aktivitätsvolumen für Drüsen mit konstanter Kerngrösse, wie die C. allata von *Leucophaea*, das bestmögliche, aus histologischen Untersuchungen ableitbare Mass für die Aktivität zu haben. Aber auch diese Grösse kann nur Indizien über die Aktivität liefern, keinesfalls aber Beweise. Die tatsächliche Aktivität der Drüse scheint sogar in vielen Fällen mehr mit den Veränderungen des Aktivitätsvolumens korreliert zu sein, als mit dessen absoluter Grösse: nach unseren bisherigen Kenntnissen müssen die C. allata in den Larvenstadien einige Zeit vor der Häutung besonders aktiv sein. Diese Phase fällt, wie Abb. 5 zeigt, nicht mit dem maximalen Aktivitätsvolumen, sondern mit dessen stärkster Zunahme zusammen. Die gleiche Feststellung wurde auch beim adulten Weibchen von *Leucophaea* inbezug auf die Wirkung der C. allata auf den Sauerstoffverbrauch gemacht (SÄGESSER, 1960). Wenn das maximale Volumen erreicht ist, ist in diesen Fällen die C. allata- Aktivität schon zurückgegangen.

Bei der Untersuchung der larvalen Entwicklung muss berücksichtigt werden, dass das Wachstum der Drüse gleichzeitig mit dem Ablauf periodischer Aktivitätsphasen erfolgt. Nach SCHARRER und VON HARNACK (1958) ist bei adulten Weibchen von *Leucophaea* ausser einer Cytoplasmazunahme auch eine Kernvermehrung mit einer Aktivierung der C. allata korreliert. Bei der Inaktivierung nimmt die Zahl der Kerne wieder ab. Es ist anzunehmen, dass auch im Zusammenhang mit den larvalen Aktivitätszyklen solche Veränderungen der Kernzahl auftreten, dass sie aber hier wegen des gleichzeitig erfolgenden Wachstums der

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Drüse nicht klar in Erscheinung treten. Die Aktivitätszyklen lassen sich deshalb als solche aus dem Verlauf der Kurven nicht immer erkennen. Werden aber die Drüsenveränderungen mit den Veränderungen des Körpergewichts in Beziehung gesetzt, wie dies in Abb. 6 geschehen ist, so lässt sich mindestens die relative Aktivität während jedes Stadiums abschätzen.

Eine Zunahme des Aktivitätsvolumens kommt bei *Leucophaea* durch Plasmavergrösserung, meist bei gleichzeitiger Kernvermehrung zustande. Aus den Abb. 2, 4 und 6 geht hervor, dass während der ersten 4 Stadien die Zellvermehrung schwach ist, der Cytoplasmazuwachs pro Zelle aber gross. Im 5. Stadium erfolgt eine vollkommene Umstellung des Wachstums: die Zellteilungsrate wird plötzlich stark erhöht (vervierfacht) und die relative Plasmamenge pro Zelle nimmt wieder ab. Auf den Zuwachs des Aktivitätsvolumens bleibt diese Umstellung im Wachstum ohne Einfluss. Seine relative Zunahme bleibt etwa gleich (Abb. 6). Es ist bis jetzt nicht ersichtlich, wie diese Wachstumsumstellung in den C. allata im 5. Stadium verursacht wird und was für eine physiologische Bedeutung sie haben könnte. Es ist jedoch interessant, festzustellen, dass bei Termiten und vielleicht auch bei anderen Insekten dem 5. Stadium eine besondere Bedeutung zukommt, da hier besonders viele neue Sinneszellen in der Epidermis entstehen (LEFEUVRE, 1959).

Aus der Kurve für das Aktivitätsvolumen in Abb. 6 ergibt sich die höchste Aktivität relativ zum Körpergewicht zu Beginn des ersten Stadiums, also unmittelbar nach dem Schlüpfen aus dem Ei. Im Laufe des ersten Stadiums sinkt das Aktivitätsplasma auf ein Minimum und steigt dann vom 2. Stadium an allmählich an. Dieser Befund deutet darauf hin, dass den C. allata wahrscheinlich schon während der Embryonalentwicklung eine inkretorische Funktion zukommt, wobei vielleicht für die erste Differenzierung der larvalen Gestalt eine besonders aktive Juvenilhormonsekretion notwendig ist.

Von manchen Autoren wird bezweifelt, dass die C. allata im letzten Larvenstadium inaktiviert werden. Nach KAISER (1949) und NOVÁK (1959) kommt es infolge eines negativ allometrischen Wachstums der C. allata notgedrungen beim Erreichen einer bestimmten Körpergrösse zu einem Stadium, indem die produzierte Juvenilhormonmenge nicht mehr genügt, um die larvale Differenzierung bei der Häutung durchzusetzen. Eine Hemmung der C. allata im letzten Stadium muss nach diesen Autoren gar nicht postuliert werden. Unsere histologischen und experimentellen Befunde widersprechen dieser Ansicht. Das durchschnittliche Zellvolumen sinkt kurz nach der letzten Larvenhäutung auf ein absolutes Minimum, und damit das Aktivitätsplasma auf Null. Das Volumen der C. allata nimmt im letzten Stadium nur sehr wenig zu, obschon die Zellteilungsrate sehr hoch ist. Jedenfalls sind die Volumenveränderungen im letzten Stadium ganz anders als in den vorangehenden Stadien und es handelt sich keineswegs um eine kontinuierliche, wenn auch negativ allometrische Grössenzunahme. Experimentell liess sich zeigen, dass die C. allata im letzten Larvenstadium gehemmt werden, und zwar auf nervösem Wege von zwei Hemmzentren im Protocerebrum aus. Werden die C. allatum-Nerven durchschnitten oder die Hemmzentren

durch eine starke Verletzung (Ausschaltung der Pericardialzellen) ausser Funktion gesetzt, so werden Mitosen in den C. allata unterdrückt, das Volumen der Drüse und das Aktivitätsvolumen bleiben jedoch gleich (Abb. 6). Dementsprechend kommt es zu extralarvalen Häutungen. Wir müssen also annehmen, dass normalerweise keine Abnahme des Aktivitätsvolumens erfolgen würde, wenn nicht im letzten Larvenstadium die nervöse Hemmung einsetzen würde. Ob das Gehirn nun die Larvenstadien 'zählt' oder von irgend einem anderen Organ im letzten Larvenstadium zu der nervösen Hemmung der C. allata veranlasst wird, wie wir anfänglich vermutet hatten, kann noch nicht entschieden werden.

Die Tatsache, dass nach Durchtrennung der C. allatum-Nerven und nach starker Verletzung die Aktivierung der C. allata von einer Hemmung der Mitoseaktivität in diesen Drüsen begleitet ist, lässt vermuten, dass mitotische Aktivität und Drüsenaktivität einander gegenseitig ausschliessen. Dies mag im letzten Stadium der Fall sein, doch trifft es für die vorangehenden Stadien nicht zu, bei denen die Zellvermehrungsrate in den C. allata trotz Aktivität gleich hoch ist wie im letzten Larvenstadium. Wahrscheinlich nimmt die durchschnittliche Zellgrösse (Abb. 2) infolge der erhöhten Zellteilungsrate vom 5. Stadium an dauernd ab und es ist möglich, dass nach Erreichen einer bestimmten Minimalgrösse ein Erhaltenbleiben der Aktivität nur noch durch Unterdrücken der Mitosen und Aktivierung des Zellwachstums möglich ist. Wir hätten dann im letzten Larvenstadium nach der Operation wieder eine Umstellung auf die erste Wachstumsart der C. allata, wie sie während der ersten 4 Stadien festgestellt wurde. Mitotische und sekretorische Aktivität schliessen sich also gegenseitig nur dann aus, wenn das durchschnittliche Zellvolumen klein ist.

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ZUSAMMENFASSUNG

(1) Als bestes histologisch auswertbares Mass der C. allata-Aktivität erweist sich das Aktivitätsvolumen. Wir verstehen darunter die Differenz zwischen dem Drüsenvolumen und dem berechneten Minimaldrüsenvolumen bei der gleichen Zellzahl.

(2) Das Aktivitätsvolumen ist nach dem Schlüpfen relativ gross, nimmt vorübergehend ab und steigt dann bis zum letzten Larvenstadium sehr stark an. Im letzten Stadium sinkt es auf Null.

(3) Die Pericardialzellen nehmen im Laufe der Entwicklung kontinuierlich an Zahl und Gesamtvolumen zu. Ein spezielles Wachstum gegen Ende der Entwicklung konnte nicht festgestellt werden.

(4) Wenn im letzten Larvenstadium kurz nach der Häutung eine bestimmte minimale Zahl von Pericardialzellen ausgeschaltet wird, so bleiben die C. allata aktiv und es kommt zu extralarvalen Häutungen.

(5) Die Wirkung dieser Operation geht über das Gehirn. Die Durchtrennung der C. allatum-Nerven zu Beginn des letzten Stadiums hat ebenfalls Aktivbleiben der C. allata und extralarvale Häutungen zur Folge.

(6) Die Implantation von Pericardialzellen in Larven des 6. und 7. Stadiums hatte keine Wirkung.

(7) Es ist wahrscheinlich, dass die in einer früheren Publikation den Pericardialzellen zugeschriebene Wirkung nicht besteht, und dass die nach Ausschaltung der Pericardialzellen beobachteten Folgen durch die Verletzung und eventuell durch Wundheilungs- und Regenerationsprozesse bedingt sind.

(8) Es konnte eindeutig nachgewiesen werden, dass die C. allata im letzten Stadium nervös vom Gehirn aus gehemmt werden, und dass ihre unterschiedliche Hormonproduktion nicht mit einem negativ allometrischen Wachstum allein erklärt werden kann.

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ANALYSIS OF SONGS OF JAPANESE CICADAS

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Abstract—Recordings have been analysed of the songs of five different species of Japanese cicadas: *Graptopsaltria nigrofuscata* Mots., *Oncotympana maculaticollis* Mots., *Tanna japonensis* Dist., *Meimuna opalifera* Walk., and *Platypleura kaempferi* F. The song was always composed of pulses of sound (damped oscillations) which are produced repetitively at a frequency of 200–500/sec by the contraction of the main sound muscle. Rhythmical changes occur in the amplitude and shape of the sound pulses. The frequency spectrum of sound shows a marked overlapping among the songs of different species but the rhythm in amplitude or shape of the pulses is specific for the species of cicada. It is concluded that species discrimination in cicadas is accomplished by discriminating the differences in these rhythms.

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AMONG the insects, sound-producing organs are best developed in the Cicadidae. The physiology of sound production has been studied for Ceylon species (PRINGLE, 1954a, b) as well as for those in Japan (HAGIWARA, 1956; HAGIWARA *et al.*, 1954; HAGIWARA and WATANABE, 1956). Recordings and analyses of the songs have also been made for the species in Ceylon (PRINGLE, 1954a, b, 1955). The present paper is concerned with the songs of five Japanese species of cicadas which were analysed on the basis of previous studies of the physiology of sound production.

MATERIALS AND METHODS

Observations were made for five species of cicadas which are common in summer in Tokyo. They were *Graptopsaltria nigrofuscata* Mot., *Oncotympana maculaticollis* Mots., *Tanna japonensis* Dist., *Meimuna opalifera* Walk., and *Platypleura kaempferi* F.

The song was first recorded on a tape (Scotch IIIA) with a tape recorder in the field. The tape recorder (modified MT-type, N.H.K. Technical Research Laboratories) and the microphone (RB-1 type ribbon microphone, N.H.K. Technical Research Laboratories) used were specially designed for recording high frequency sounds. Frequency analysis of sound was performed from the tape by using the sonagraph (new type, Key Electronic Company); oscillographic recording of sound was also done from the tape through a cathode-ray oscilloscope (DuMont Type 333). With the use of the equalizer the present recording system showed a flat response between 1 kc and 40 kc within 3 dB range.

The recordings of impulse discharge of the muscle and of the sound produced by the activity of the same muscle were made in a soundproof room; the further techniques for impulse recordings were similar to those employed previously (HAGIWARA, 1956).

RESULTS

The song of a cicada is produced by the vibration of the membrane called the drum membrane. The vibration is initiated and maintained by the activity of the main sound muscle which is connected to the membrane through a fine tendon. Figs. 1–4 show the sound and simultaneously recorded impulse discharge of the main sound muscle obtained during the shrill (short cry induced by handling) of four different species. Since the main sound muscle is innervated by a single motor nerve fibre, each impulse recorded indicates the arrival of a motor impulse at the muscle. These recordings were obtained after the contralateral main sound muscle had been destroyed; they therefore show the relations between the motor impulse arriving at the muscle and the sound produced by the same muscle. The relations are different from species to species. In *Graptopsaltria nigrofuscata* (Fig. 1), *Oncotympana maculaticollis* (Fig. 2), and *Tanna japonensis* each motor impulse results in a rapid twitch of the muscle and a pulse of sound (a damped oscillation of the drum membrane) is then produced. In some species such as *Oncotympana maculaticollis* a single twitch sometimes produces two successive pulses; these are probably due to 'In and Out clicks' reported for Ceylon cicadas (PRINGLE, 1954). In *Meimuna opalifera* (Fig. 4) the number of sound pulses corresponding to each motor impulse is not always one but sometimes it is as many as ten or more. However, the correspondence between the impulse and the sound pulse is still observed in this species. In contrast to this no obvious correspondence is found in *Platypleura kaempferi*, as shown by Fig. 3. As reported previously (HAGIWARA, 1954) the tension of the drum membrane is regulated by the activity of the accessory sound muscle and this results in changes of shape and amplitude of each damped oscillation.

The sound produced by the vibration of the drum membrane is modified by the 'resonant system'. The characteristics of this system are changed by activity of the abdominal muscles, especially the intersegmental regulating muscles; the important changes are in the volume of the abdominal air chamber and in the tension of joint membranes which cover the window of the resonant chamber. A protuberance, found in front of each joint membrane and called the abdominal valve, closes or opens this window by movement of the abdomen relative to the thorax.

The complex rhythms observed in the songs of cicadas are due to complex activities of these muscles. In the following sections the song of each species will be described.

1. *Graptopsaltria nigrofuscata* (Fig. 5, A)

The song of this species consisted of a cycle lasting 20–40 sec. At the beginning of this cycle the song was composed of bursts of pulses, the bursts repeating at approximately 12/sec throughout the song cycle. At the beginning the bursts had a relatively short duration, but this increased rapidly until the silent period between successive bursts completely disappeared. In this continuous phase of the song (Fig. 5 (i), A1) the rhythm at approximately 12/sec still remained

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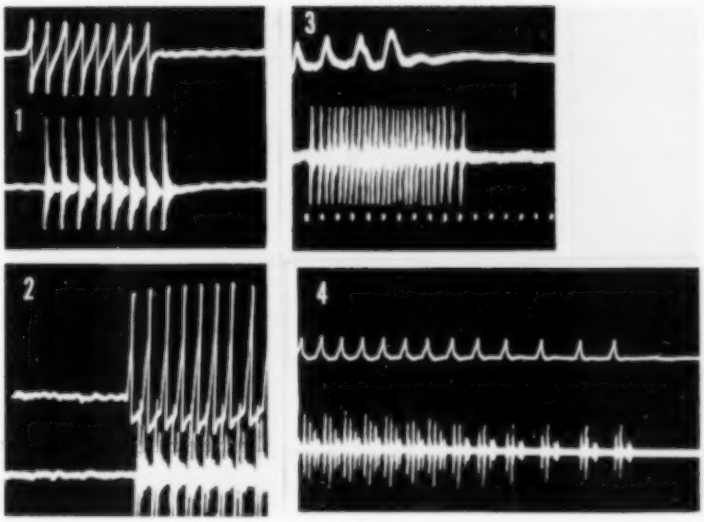

FIGS. 1–4. Correlation of impulse discharge in the main sound muscle (upper trace) and the recorded sound (lower trace). The contra-lateral main sound muscle had been destroyed. Time, 10 msec. (1) *G. nigrofuscata*. (2) *O. maculaticollis*. (3) *P. kaempferi*. (4) *M. opalifera*.

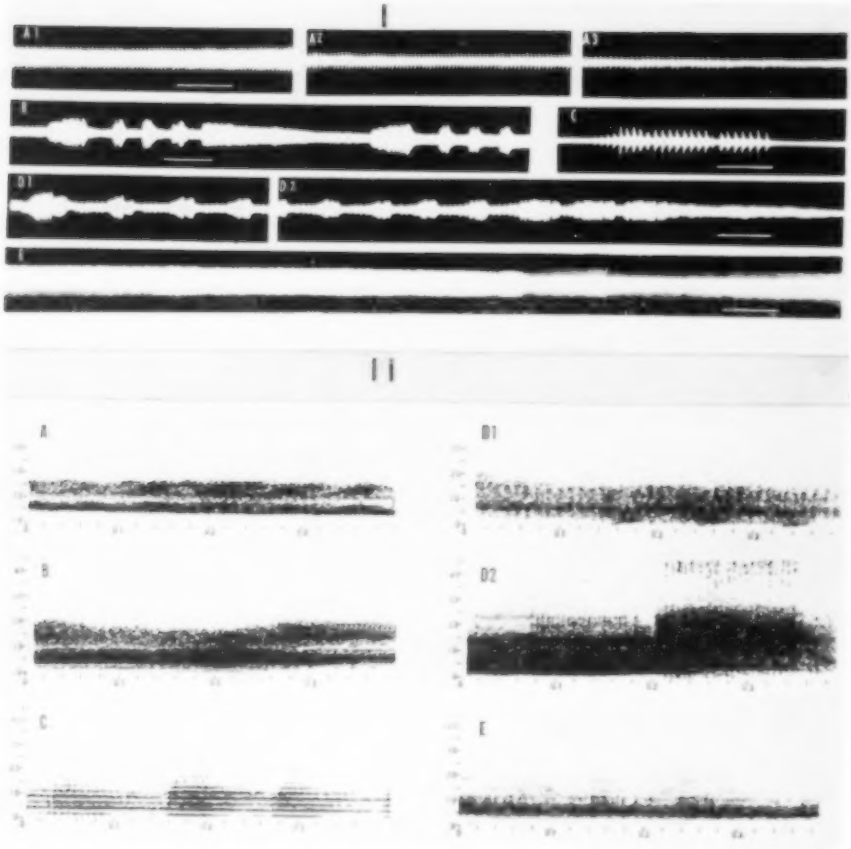

FIG. 5. (i) Low speed oscillograms of the song patterns of cicadas. Time, 1 sec.
(ii) Sonograms of the songs of cicadas. Ordinates, frequency of sound in kc/s. Abscissae, time in seconds.

A, *Graptopsaltria nigrofuscata*. B, *Oncotympana maculaticollis*. C, *Tanna japonensis*. D, *Meimuna opalifera*. E, *Platypleura kaempferi*.

as a rhythm of amplitude modulation of the sound pulses. The continuous phase was followed again by a phase with discrete bursts (Fig. 5 (i), A2), the duration of which gradually decreased until sound production ceased. Within the discrete bursts and during the continuous phase the pulses repeated at a frequency at approximately 200/sec. Since the main sound muscles receive motor nerve impulses alternatively in this species (HAGIWARA, 1956), the present observations indicate that motor nerve impulses arrive at each muscle at a fairly constant frequency (about 100/sec) during the prolonged cycle of song as during the shrill (HAGIWARA, 1956). The changes of amplitude of the pulses within each burst and during the continuous phase were probably due to activity of the accessory sound muscle which is highly developed in this species. The sonagram (Fig. 5 (ii), A), made during the continuous phase of the song, shows that a rhythmic increase of high frequency components accompanies the rhythmic increase in amplitude of the sound pulses.

2. *Oncotympana maculaticollis* (Fig. 5, B)

The song of this cicada was an uninterrupted series of sound pulses, the amplitude of which was modulated with a slow rhythm. The pulse frequency was constant at around 200/sec. The relation between the pulse of sound and the motor impulse arriving at the main sound muscle similar in this species to that in *Graptopsaltria nigrofuscata*. The frequency of motor impulses during the song of both species was constant at approximately 100/sec. However, in the present species single motor impulses sometimes produced two pulses of sound; this makes the sound recording less simple than in *Graptopsaltria nigrofuscata*. The alteration of the large and small amplitude phases were associated with the corresponding abdominal movement of the cicada. By this movement the abdominal valves of the insect closed the joint membranes during the small amplitude phase while they were exposed during the large amplitude phase. Fig. 5 (ii), B1 is a sonagram during a brief large amplitude phase of the song; it shows that high frequency sound components increased in the small amplitude phase. This result is in a contrast to the increase of high frequency components associated with the large amplitude phase in *Graptopsaltria nigrofuscata*.

3. *Tanna japonensis* (Fig. 5, C)

The song of this cicada was a series of bursts of pulses, bursts repeating at approximately 4–6/sec. The total number of bursts in one cycle of song ranged between fifteen and twenty-five. The sonagram (Fig. 5 (ii), C) comprises three bursts of pulses; this record shows that a simple relationship holds between the fundamental and the higher harmonics in the sound spectrum just as usually observed for a musical instrument. No such simple relations were observed for other species of cicadas. The song of this species is generally believed to be the most musical.

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4. *Meimuna opalifera* (Fig. 5, D)

The song of this species showed the most complex pattern of sound production among those examined in the present programme. The song was characterized by the alternation of two phases, one of a larger amplitude and one of smaller but rhythmically increasing amplitude. Since the relation between motor impulses and sound pulses is not simple in this species the pattern of motor impulse discharge could not be deduced from analysis of the recorded sound. The rhythmical change in the song was accompanied by corresponding movements of the insect's abdomen; the abdominal regulating muscles are well developed in this species.

In Fig. 5 (ii), D1 and D2 are shown sonagrams respectively in the small and large amplitude phases of the song; there is an enormous increase of high frequency components during the large amplitude phase. The frequency of the most dominant sound component was about 7 kc in the phase of oscillatory amplitude and also in the beginning of the large amplitude phase but it increased during the latter, finally reaching a value as high as 20 kc. The highest frequency observed for the song of this species was 25 kc and this was the maximum frequency of sound observed in the present programme.

5. *Platypleura kaempferi* (Fig. 5, E)

The song of this cicada was a simple sequence of sound pulses, repeating at 400–500/sec. However, this does not indicate the frequency of motor impulses arriving at the main sound muscle since no regular relation is found between impulses and the sound pulse in this cicada (HAGIWARA *et al.*, 1954). Although the amplitude of the pulses remained unchanged during the whole cycle of the song a marked change appeared in the rate of damping. At the beginning of the song the rate of damping was very small but it showed a gradual increase up to a certain maximum. This was followed by a sudden decrease of the rate of damping and then again a gradual increase occurred. Such an alternation was repeated indefinitely. These changes were associated with the movement of the insect abdomen. The sound spectrum showed no significant changes during the phases of high or low damping.

DISCUSSION

Although the frequency ranges of the sonagrams show some variations among the songs of different species a marked overlapping is found for the whole range of spectrum. The frequency range of the dominant sound components is 5–7 kc. Sound-receiving mechanisms of cicadas have recently been analysed by recording impulse discharges of the acoustic nerve to pulses of varying frequencies (KATSUKI and SUGA, 1959, 1960). The frequency range of sound to which the receptor system was most sensitive was 4–5 kc and was relatively independent either of the species or the sex of specimen used (*G. nigrofuscata*, *T. japonensis*, *P. kaempferi*, and *M. opalifera*). This range coincides with that of dominant sound components in the songs and it may be concluded that cicadas are very sensitive to their

own songs. However, because of the overlapping frequency ranges mentioned above it is very unlikely that the species discrimination of the cicada is performed through the analysis of the frequency spectrum of the song by the receptor system. In addition to this, the sound receptor of insects are probably incapable of analysing sound frequencies. This is because the sensitive frequency ranges of sounds are the same for all the examined nerve fibres in the acoustic nerve (KATSUKI and SUGA, 1959, 1960).

The song of a cicada is mainly characterized by the rhythm of the amplitude change or sometimes of the change of rate of damping in damped oscillations. These rhythms are very specific to the species of cicada. Furthermore, it has been shown that the change of intensity in applied sound is very readily detected by the cicada receptor (KATSUKI and SUGA, 1959, 1960). In other words the receptor system of the cicada is well adapted for the discrimination of these rhythms. From these results it is concluded that species discrimination in cicadas is performed through the differences in the rhythms of their songs.

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ÜBER DIE WIRKUNG DER CORPORA ALLATA AUF DEN SAUERSTOFFVERBRAUCH BEI DER SCHABE *LEUCOPHAEA MADERAE* (F.)*

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Abstract—The oxygen consumption in adult females of *Leucophaea maderae* is correlated to the reproductive cycle and to the cyclic changes in volume of the corpora allata. The maximum O_2-consumption is reached during the phase of most rapid increase in volume of the c. allata.

If the ootheca of pregnant females is removed, the c. allata increase in size. The O_2-consumption also increases and reaches the value of the maximum of the normal cycle. Castration is also followed by an increase of O_2-consumption.

In order to test the influence of the c. allata on O_2-consumption, active and inactive c. allata were implanted in castrated females. The increase of O_2-consumption could not be due to the activity of the ovaries and of the accessory glands, which had in some experiments been removed together with the ovaries. The implantation of active c. allata from donors which had deposited their eggs 4 days before, was followed by a significant increase in O_2-consumption. The implantation of inactive c. allata taken from pregnant females and the injection of Ringer solution had no effect. It is evident therefore that a hormone of the c. allata has a stimulatory effect upon the respiratory metabolism.

During the two last larval instars respiration follows essentially the same course although a difference in the juvenile hormone secretion of the c. allata has to be postulated in these instars (inactivation during the last instar brings about metamorphosis). The hormone which stimulates the respiratory metabolism can therefore not be identical with the juvenile hormone, whereas its identity with the gonadotropic hormone of the c. allata is probable. The c. allata therefore seem to produce at least two different hormones: a juvenile hormone and a gonadotropic hormone.

I. EINLEITUNG

Bei verschiedenen Insekten produzieren die Corpora allata adulter Weibchen ein Hormon, das für die Eireifung, beziehungsweise für die Dottereinlagerung in die Oocyten notwendig ist (Wigglesworth, 1936; Weed (Pfeiffer), 1936; Scharrer, 1946 uam.). Überdies konnte gezeigt werden, dass das Corpora allata-Hormon bei *Leucophaea* eine stimulierende Wirkung auf die Sekretion der Anhangsdrüsen des weiblichen Geschlechtsapparates hat (Scharrer, 1946; Engelmann, 1957a), dass es bei *Melanoplus* den Fettkörper beeinflusst (Pfeiffer, 1945), und dass es bei *Dixippus* für die Beinregeneration notwendig ist (Pflugfelder, 1939). Neben

* Dissertation (Leitung: Prof. Dr. M. Lüscher).

der gonadotropen Wirkung im adulten Weibchen scheint also das Corpora allata-Hormon noch eine allgemein stimulierende Wirkung auf den Metabolismus zu haben.

Diese Wirkung äussert sich im Sauerstoffverbrauch: THOMSEN (1949) beobachtete bei *Calliphora erythrocephala* nach Corpora allata-Exstirpation eine Senkung des Sauerstoffverbrauchs bei beiden Geschlechtern, nach Corpora allata-Implantation eine Steigerung bei Weibchen, jedoch keine Wirkung bei Männchen. PFLUGFELDER (1952) fand, dass bei *Dixippus* nach Corpora allata-Exstirpation der Sauerstoffverbrauch vorübergehend gesenkt, nach Implantation dagegen während kurzer Zeit erhöht wird. Der Autor schliesst daraus und aus den negativen Ergebnissen bei Corpora allata-Implantation in *Calliphora*-Männchen (THOMSEN, 1949) auf eine indirekte Wirkung des Hormons, bedingt durch die Dotterbildung im Ovar. Kastration bewirkt jedoch bei Weibchen von *Calliphora* keine Veränderung des Sauerstoffverbrauchs (THOMSEN und HAMBURGER, 1955). Da in diesem Falle das Ovar als Verbraucher ausgeschaltet war, schliessen die Autorinnen auf eine direkte Wirkung des Corpora allata-Hormons auf den Stoffwechsel. NOVÁK, SLÁMA und WENIG fanden bei kastrierten Weibchen von *Pyrrhocoris* keine Steigerung des Sauerstoffverbrauchs nach Implantation von Corpora allata, woraus sie wie PFLUGFELDER auf eine indirekte Wirkung des Corpora allata-Hormons schliessen.

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Die Frage der Stimulierung des respiratorischen Stoffwechsels durch das Hormon der Corpora allata ist also sehr umstritten und bedarf einer weiteren Untersuchung. Die sich zum Teil widersprechenden Ergebnisse bei Implantationsversuchen könnten vielleicht darauf zurückzuführen sein, dass die Funktionszyklen nicht bekannt waren und deshalb nicht berücksichtigt werden konnten.

Zur Abklärung der Frage der direkten Stimulierung des Stoffwechsels durch das Hormon der Corpora allata ist die ovovivipare Schabe *Leucophaea maderae* besonders geeignet, da sie einen mit der Ovarfunktion zusammenhängenden Corpora allata-Zyklus aufweist, welcher von ENGELMANN und LÜSCHER (1955–1960) eingehend untersucht worden ist. Es ist somit möglich, aktive oder inaktive Drüsen zu implantieren. Auch die Messung des Sauerstoffverbrauchs über den ganzen Entwicklungszyklus erlaubt es, Zusammenhänge ohne jegliche Operationen aufzuzeigen. Die Versuchstiere besitzen überdies den Vorteil, dass sie recht gross und zählebig sind. Es ist möglich, die Weibchen im letzten Larvenstadium zu kastrieren und im Adultstadium für Implantationsversuche zu verwenden (PFLUGFELDER, 1952, p. 248).

In der vorliegenden Arbeit wird untersucht, ob zwischen Corpora allata-Volumen und Sauerstoffverbrauch eine Korrelation besteht. Ferner wird durch Implantation aktiver und inaktiver Corpora allata in kastrierte Weibchen die Frage der direkten stoffwechselstimulierenden Wirkung analysiert. Durch Vergleich der Stoffwechselwirkung mit den andern Wirkungen der Corpora allata soll schliesslich untersucht werden, ob das den respiratorischen Stoffwechsel stimulierende Hormon mit dem gonadotropen Hormon und mit dem Juvenilhormon identisch sein kann.

II. MATERIAL UND METHODEN

Das verwendete Tiermaterial stammte aus der Zucht des Zoologischen Instituts Bern.* Je zwei Männchen und Weibchen wurden in Gläsern von 1 Liter Inhalt bei 26°C und 75–80% rel. Luftfeuchtigkeit gehalten und mit Hundekuchen, Karotten und Äpfeln gefüttert (ENGELMANN, 1957b). Die tägliche Kontrolle lieferte die Daten der Häutung, Ovulation und der Ablage der Larven (LÜSCHER und ENGELMANN, 1955; ENGELMANN, 1957a). Die errechneten und für diese Darstellung verwendeten Durchschnittswerte liegen meist etwas tiefer als die von ENGELMANN (1957b) angegebenen.

TABELLE 1—DURCHSCHNITTLICHE DAUER DER EINZELNEN STADIEN

	Tage
Larven des 7. Stadiums	34
Larven des 8. Stadiums	38
1. Eireifungsperiode	28
1. Trächtigkeitsperiode	75
2. Eireifungsperiode	19

Zur Durchführung der Messungen des Sauerstoffverbrauchs erwies sich eine modifizierte Form des Mikro-Respirationsapparates von KRÜGER (1935) als geeignet, da durch die Pumpwirkung des KOH-Gefässes die Atemluft ständig im Umlauf gehalten und dadurch eine praktisch vollständige, gleichmässige Absorption erreicht wird.

Der Apparat (Abb. 1) besteht aus einem geschlossenen Kreislauf von Atmungsgefäss *A* und Pumpe *B*, welche zugleich Absorptionsgefäss ist. Durch eine Pendelbewegung senkrecht zur Bildebene, wobei die Drehachse ungefähr durch den Dreiweghahn *C* zwischen Atmungsgefäss und Pumpe zu denken ist, wird die Apparatur geschaukelt. Die Pumpe ist bis zum gestrichelten Niveau mit KOH (33%) gefüllt. Bei jeder Pendelbewegung perlen einige Luftblasen von *D* nach *E* durch die KOH-Lösung. Bei unseren Pumpen beträgt die Pendelzeit ungefähr 2 sec.

Zur bequemeren Handhabung befinden sich die Tiere in einem Käfig aus perforiertem, durchsichtigem Celluloid (bei hohen Extremwerten kann durch Beobachtung festgestellt werden, ob sich die Tiere bewegen).

Vier solcher Apparate sind durch die Verteilleitung *J*, an welcher sie mit Normalschliffen angeschlossen sind, mit dem Manometer *K* verbunden. Alle Leitungen ausserhalb des Wasserbades *L* sind aus Kapillarrohr hergestellt, um das unter Aussentemperatur stehende Volumen möglichst niedrig zu halten. Der andere Manometerschenkel ist mit dem Kompensationsgefäss *M* verbunden; dadurch wird der Einfluss des Barometerdrucks ausgeschaltet.

Da das ausgeatmete CO_2 absorbiert wird, entsteht im Kreislaufsystem ein Unterdruck wegen des O_2-Verbrauchs der Versuchstiere. Nacheinander wird durch Öffnen der Hahnen *N* dieser Unterdruck auf das Manometer übertragen. Statt nun die Manometerwerte in Volumenanteile umzurechnen, wird die Quecksilbersäule *O* mittelst des Quetschhahns *P* so hoch in das auf 10μl geeichte, kalibrierte Rohr *Q* getrieben, dass das Manometer wieder auf dem ursprünglichen Wert steht. Die verbrauchte O_2-Menge wird dadurch ersetzt und kann direkt abgelesen werden mit einer Ablesegenauigkeit von $\pm 5\mu$l.

Alle bis jetzt beschriebenen Teile sind aus Pyrexglas angefertigt und durch Schliffe starr miteinander verbunden. Manometer und Quecksilbersäule bewegen sich also beim Pendeln mit.

Der Dreiweghahn *R* gestattet, die Quecksilbersäule wieder zurückzustellen. Er ist durch einen Schlauch *S* mit dem Gasometer *T* verbunden, in welches aus einer Bombe O_2 eingefüllt wird. Das Rohr *Q* und die Verteilleitung *J* sind mit O_2 gefüllt, bei jeder Messung

* Ursprünglich aus der Zucht von Frau Dr. B. Scharrer, New York.

wird soviel O_2 in die Kreislaufsysteme gebracht wie verbraucht wurde, die Atemluft behält also ihren ursprünglichen O_2-Gehalt. Man kann allerdings trotzdem nicht beliebig lange messen, da die Versuchstiere durch die konzentrierte KOH-Lösung ausgetrocknet werden.

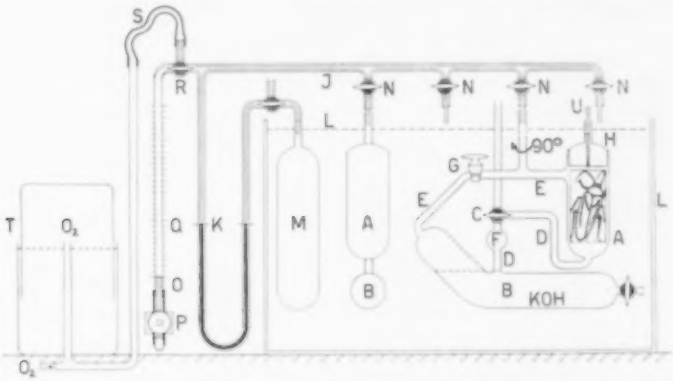

ABB. 1. Respirationsapparat mit Atmungsgefäss A, Pumpe B, Manometer K, Wasserbad L, Ballon zum Auffangen der KOH-Spritzer F, Hahn zum Abschalten der Pumpe G, Deckel H (weitere Erläuterungen im Text).

Die ganze Apparatur befindet sich in einem Wasserbad von 26°C (normale Zuchttemperatur). Vor jeder Messung muss sich die Luft im Atmungsgefäss auf diese Temperatur erwärmen. Erfahrungsgemäss dauert das ungefähr eine Stunde bei laufender Pumpe. Die Prüfung erfolgt durch Thermoelemente *U*, welche in den Deckel jedes Atmungsgefässes eingeschmolzen sind. Wenn das angeschlossene Galvanometer gegenüber einem ins Wasserbad gehängten Vergleichselement keinen Ausschlag zeigt, fliesst kein Strom, dh. beide Temperaturen sind gleich. Gleichzeitig wird die Luft vor Beginn der Messung durch das Pendeln vom CO_2 befreit.

Da sich *Leucophaea* gerne in engen Röhren verkriecht, verhält sie sich im Atmungskäfig meist vollständig ruhig. Während der Messungen aktive Tiere wurden nicht gewertet. Ein tageszeitlicher Aktivitätsrhythmus konnte im Gegensatz zu *Periplaneta americana* (HARKER, 1956) bei *Leucophaea* nicht festgestellt werden.*

Es wurden jeweils drei Messungen, je während 20 Minuten, durchgeführt. Differierten die Werte um mehr als 10%, so wurde weiter gemessen, und die höchsten Werte wurden nicht berücksichtigt. Dies war aber selten notwendig. Nach den Messungen wurde das Gewicht der Versuchstiere bestimmt. Die Werte betreffen ausschliesslich Einzeltiere. Mehrere Tiere in einem Gefäss bewegen sich oft und liefern unzuverlässige Werte. Die Angabe des O_2-Verbrauchs erfolgt stets in Millilitern pro Gramm und pro Stunde (ml g^{-1} h^{-1}).

Alle Operationen wurden unter CO_2-Narkose durchgeführt. Durch Fingerdruck auf das Abdomen trächtiger Weibchen lässt sich das Eipaket leicht entfernen. Anschliessend muss der herausgestülpte Uterus mit einer stumpfen Pinzette wieder in die Vagina geschoben werden (ENGELMANN, 1957b); andernfalls dorrt das heraushängende Stück ab, und eine neue Ovulation erfolgt verspätet.

Die Ovarien wurden im letzten Larvenstadium entfernt, indem die Flankenhaut des 6. Segmentes beidseitig aufgetrennt und durch leichtes Drücken etwas Fettkörper herausgepresst wurde, in welchem die Ovarien eingebettet liegen. Diese wurden mitsamt dem

* Dies dürfte darauf zurückzuführen sein, dass unsere Versuchstiere dauernd im Dunkeln gehalten werden.

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Fett abgetrennt. Alle Tiere überlebten die Operation, und die Sektion nach den Messungen zeigte, dass die Ovarien stets vollständig fehlten. Die Entfernung des Uterus und der Anhangsdrüsen erfolgte durch Herausziehen und Abschneiden von der Geschlechtsöffnung her. Die Adulthäutung wickelte sich 2 bis 39 Tage nach der Operation normal ab. Die O_2-Messungen wurden frühestens 13 Tage nach dem Eingriff durchgeführt.

Die für die Implantationsversuche benötigten C. allata wurden in Ringerlösung herauspräpariert und mit Hilfe einer Injektionsspritze mit Glasnadel in den Rücken des Abdomens injiziert.

Da es sich bei der statistischen Auswertung stets um die Prüfung der Differenz zwischen zwei Mittelwerten oder zwischen den Steigungen zweier Regressionen handelte, wurde der *t*-Test von 'Student' (LINDER, 1951) angewendet. Die Mittelwerte wurden berechnet aus der Summe der Durchschnitte aller Messungen des gleichen Tieres, denn es war oft nicht möglich, jedes Tier gleich oft zu messen. Dabei wurden allerdings vereinfachend die einzelnen Durchschnitte nicht mit der reziproken Streuung multipliziert, so dass nun die Anzahl der Messungen pro Tier nicht berücksichtigt ist; jedes Tier hat also statistisch das gleiche 'Gewicht'.

Die in den Abbildungen eingezeichneten Kurven entsprechen dem sog. 'gleitenden Durchschnitt'.

III. DER ZYKLUS DER CORPORA-ALLATA-AKTIVITÄT UND DES SAUERSTOFFVERBRAUCHS

a. Der Sauerstoffverbrauch adulter Weibchen

Der untersuchte Normalzyklus umfasst die erste Eireifungsperiode bis zur ersten Ovulation, die erste Trächtigkeitsperiode bis zur Ablage und die zweite Eireifungsperiode (LÜSCHER und ENGELMANN, 1955; ENGELMANN, 1957a). Als Eireifung wird hier das Wachstum der Oocyten unter Dottereinlagerung bezeichnet. Das C. allata-Hormon wird deshalb oft auch Dotterbildungshormon genannt. Entgegen der von diesen Autoren publizierten durchschnittlichen Dauer der einzelnen Perioden von 35, 75, und 25 Tagen erhielt ich 29 Tage für die erste Eireifungsperiode, 75 Tage für die Trächtigkeit und 20 Tage für die zweite Eireifung. Möglicherweise hängt dies mit verbesserten Zuchtbedingungen zusammen (konstante Feuchtigkeit). Die in den vorgenannten Arbeiten publizierte Kurve des C. allata-Volumens wurde auf meine Werte umgezeichnet (Abb. 2): In der ersten Eireifungsperiode wird nun ein Maximum des C. allata-Volumens am 15. Tag erreicht, daraufhin erfolgt ein kontinuierliches Absinken bis in die Mitte der Trächtigkeitsperiode, dann nimmt das Volumen zuerst langsam, bei der Ablage aber sehr rasch zu, bis am 12. Tag ein Maximum erreicht wird, das 2½-mal so hoch ist wie das Maximum der ersten Eireifungsperiode.

Spiegelbildlich zu dieser Kurve verläuft die Kurve der Kerndichte (LÜSCHER und ENGELMANN, 1955; ENGELMANN, 1957a). Das Volumen der Drüsen darf nach ENGELMANN (1957a) als Mass für die Aktivität gelten.

Die Kurve des Sauerstoffverbrauchs (Abb. 2) stellt den gleitenden Durchschnitt aus 249 Messungen dar, wobei einzelne Weibchen während des ganzen Zyklus, andere nur während kürzerer Perioden gemessen wurden. Die gleichen Tiere wurden in Abständen von 2 bis 10 Tagen gemessen. In keinem Bereich der Kurve stammen die Werte von weniger als von 8 Weibchen. Da die Dauer der

Perioden individuell verschieden ist, wurden die Werte der einzelnen Kurven dem Durchschnitt entsprechend proportional zusammen- oder auseinandergezogen.

Der Sauerstoffverbrauch ist bei der Adulthäutung schon sehr hoch (vgl. Abb. 7, Sauerstoffverbrauch im letzten Larvenstadium). Er steigt noch und erreicht am 9. Tag ein Maximum von 0,423 ml O_2 $g^{-1}h^{-1}$, um dann rasch

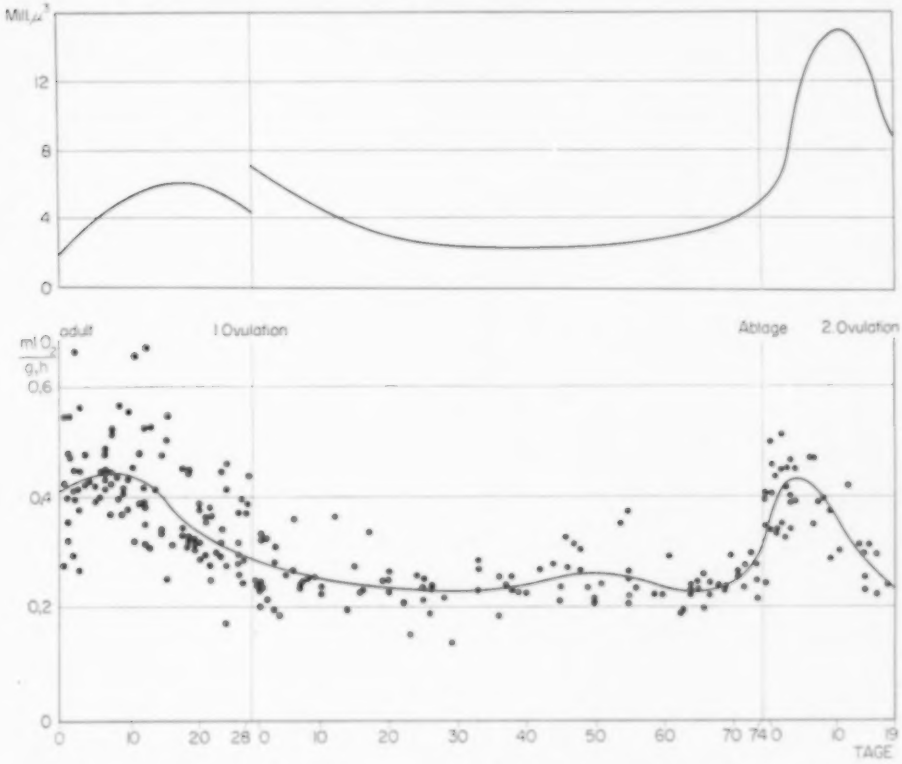

ABB. 2. Oben: C. allata-Volumen von der Adulthäutung bis zur 2. Ovulation, nach ENGELMANN, verändert. Ordinate: Volumen in Millionen μ^3. Unten: Veränderungen im Sauerstoffverbrauch während derselben Zeit. Ordinate: O_2-Verbrauch in ml pro g Lebendgewicht und Stunde. Abszisse: Dauer der einzelnen Stadien in Tagen.

abzusinken. Während der Trächtigkeit wird eine Phase erreicht, in welcher der O_2-Verbrauch nur wenig schwankt. Aus den Werten zwischen dem 15. und 65. Tag wurde ein Durchschnittswert des Normalverbrauchs von 0,247 ml O_2 $g^{-1}h^{-1}$ bestimmt. Vom 66. Tag der Trächtigkeitsperiode an steigt der Verbrauch und erreicht am 4. Tag nach der Ablage ein Maximum von 0,425 ml O_2 $g^{-1}h^{-1}$.

Bei Einzeltieren können gewisse Schwankungen auftreten, doch ergibt sich immer dasselbe Gesamtbild. Abb. 3 zeigt zwei solche Kurven: Sie beginnen mit der Adulthäutung. Die Ovulationen und Ablagen stimmen zeitlich nicht überein.

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Die eine Kurve zeigt die Werte des Weibchens mit den höchsten Maxima; sie verläuft meist über der andern Kurve, welche dem Normalfall entspricht.

Die Kurven der C. allata-Volumina und des O_2-Verbrauchs (Abb. 2) zeigen eine gewisse Übereinstimmung, jedoch werden die beiden O_2-Maxima einige Tage vor den Volumenmaxima erreicht. Wenn wir von der Annahme ausgehen, dass

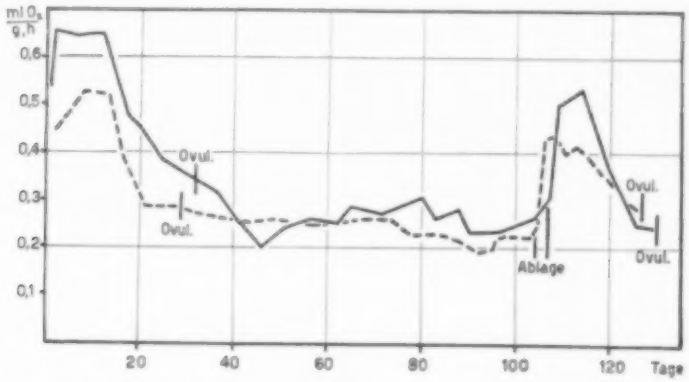

ABB. 3. Sauerstoffverbrauch zweier Weibchen. Die Kurven beginnen mit der Adulthäutung. Die ausgezogene Linie stellt den Verbrauch eines Tieres mit sehr hohen Extremwerten dar, die gestrichelte Kurve dagegen entspricht ungefähr dem Normalfall.

der Versuch eine Abhängigkeit des Stoffwechsels von der Tätigkeit der C. allata zeigt, dann muss die Ausschüttung eines stoffwechselstimulierenden Hormons dann am stärksten sein, wenn die Drüse am stärksten anwächst, da das O_2-Maximum im Bereich des steilsten Anstiegs der Volumenkurve liegt. Es ist bemerkenswert, dass die O_2-Maxima gleich hoch sind, dass das zweite Volumenmaximum aber viel grösser ist als das erste. Der O_2-Verbrauch verändert sich also nicht proportional zum Drüsenvolumen. Es fragt sich deshalb, ob das Volumen der C. allata immer der Aktivität entspricht.

Auf Grund der Ergebnisse kann jedoch nicht zwingend auf eine direkte Wirkung der C. allata auf den O_2-Verbrauch geschlossen werden, denn parallel zur Aktivität der C. allata und von diesen gesteuert verläuft der Zyklus der Ovar- und Anhangsdrüsentätigkeit. Die Schwankungen im O_2-Verbrauch hängen möglicherweise hiervon ab. Die Wirkung der C. allata auf den O_2-Stoffwechsel wäre dann nur indirekt.

b. Der Sauerstoffverbrauch adulter Männchen

Bei adulten Männchen von Insekten ist nach den bisherigen Kenntnissen die Gonadentätigkeit unabhängig von den C. allata. Das Verhalten der C. allata ist demgemäss einfach (SCHARRER und v. HARNACK, 1958): Nach der Adulthäutung steigt das Volumen von ungefähr 3 Mio μ^3 auf 8 Mio am 10. Tag, wobei allerdings die Kernzahl evenfalls ansteigt, wenn auch nur halb so stark. Nach einem Abfall

schwankt das Volumen vom 25. Tag an um ein Mittel von 5 Mio μ^3, doch werden diese Schwankungen von der Kernzahl mitgemacht, so dass sich das Zellvolumen nicht mehr ändert.

Acht Männchen wurden währen 12 Tagen gemessen. Die Kurve des Sauerstoffverbrauchs (Abb. 4) beginnt mit einem Maximum von 0,404 ml O_2 $g^{-1}h^{-1}$ bei der Häutung und sinkt nach 2–3 Tagen auf einen konstanten Wert, der ungefähr gleich hoch ist, wie der nach 60 Tagen aus 7 Tieren durch einmaliges Messen bestimmte Wert von $0{,}324 \pm 0{,}007$ ml O_2.

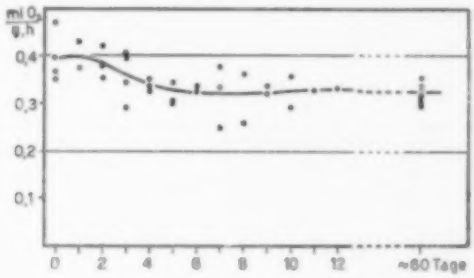

ABB. 4. Sauerstoffverbrauch der Männchen, von der Adulthäutung an gemessen.

Auch hier läuft das Maximum des Sauerstoffverbrauchs dem C. allata-Volumenmaximum voraus. Es befindet sich ebenfalls in der Zone der stärksten Volumenzunahme. Eine klare Korrelation zwischen O_2-Verbrauch und C. allata-Volumen ergibt sich daher bei Weibchen und Männchen nur in der Phase des Minimalverbrauchs (Tab. 2): Die trächtigen Weibchen sind schwerer als die Männchen, sie haben kleinere C. allata und brauchen demgemäss weniger Sauerstoff. Die Differenz des Sauerstoffverbrauchs ist gesichert mit $P < 0{,}002$.

TABELLE 2—VERGLEICH DES GEWICHTS, DES C. ALLATA-VOLUMENS UND DES SAUERSTOFFVERBRAUCHS VON MÄNNCHEN UND TRÄCHTIGEN WEIBCHEN

	Männchen	Trächtige Weibchen
Gewicht in g, aus je 10 Stichproben	$1{,}4 \pm 0{,}06$	$2{,}37 \pm 0{,}07$
Volumen der C. allata in Mio μ^3	4,2*	2,5†
O_2-Verbrauch in ml $g^{-1}h^{-1}$	0,324	0,247

* Nach SCHARRER und v. HARNACK (1958).

† Nach ENGELMANN (1957a).

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IV. DER SAUERSTOFFVERBRAUCH UNTER EXPERIMENTELLEN BEDINGUNGEN

a. Die Wirkung der Entfernung des Eipakets

Nach ENGELMANN (1957a) hat das Eipaket im Uterus eine hemmende Wirkung auf die Aktivität der C. allata. Wird es experimentell entfernt, dann werden die C. allata aktiv und die Oocyten im Ovar beginnen zu wachsen. 15–17 Tage nach der Operation zeigt sich eine Aktivität, die—gemessen an dem Volumen der Drüsen und an der Kerndichte—stark über den Werten der Trächtigkeitsperiode liegt, aber das nomale Maximum, wie es 14 Tage nach der Ablage auftritt, nicht erreicht. ENGELMANN glaubt diese Differenz durch Hungern, hervorgerufen durch die Operation, erklären zu können, da Hungerversuche für diese Interpretation sprachen.

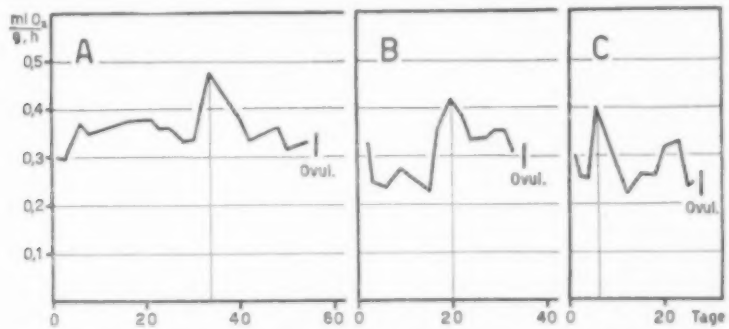

ABB. 5. Sauerstoffverbrauch nach experimenteller Entfernung des Eipakets. A: Eipaket 3 Tage nach Ovulation entfernt, O_2-Maximum nach 34 Tagen. B: Eipaket 20 Tage nach Ovulation entfernt, O_2-Maximum nach 20 Tagen. C: Eipaket 37 Tage nach Ovulation entfernt, O_2-Maximum nach 6 Tagen.

Um zu prüfen, ob auch in diesem Falle der O_2-Verbrauch mit zunehmendem C. allata-Volumen ansteigt, wurde bei 18 trächtigen Weibchen im Alter von 3 bis 55 Tagen nach der ersten Ovulation das Eipaket entfernt. Bei 6 Tieren kam es zu keiner neuen Ovulation, wahrscheinlich, weil bei der 1. Ovulation Eier im Ovar zurückgeblieben waren, und die C. allata daher weiterhin gehemmt waren (ENGELMANN, 1957a). 12 Weibchen ovulierten nach 26 bis 56 Tagen. Während dieser Zeitspanne wurde alle 2 oder 3 Tage der O_2-Verbrauch festgestellt. Dieser blieb nach der Operation anfänglich niedrig (Abb. 5), um dann für einige Tage anzusteigen und wieder auf das alte Niveau abzusinken. Abgesehen von einer verschieden lang dauernden Phase vor Erreichen des Maximums mit mehr oder weniger konstantem O_2-Verbrauch entsprechen die Kurven den in Abb. 3 gezeigten Einzelkurven zwischen Ablage und neuer Ovulation. Je früher nach der Ovulation die Operation durchgeführt wird, desto länger dauert es, bis das Maximum des Sauerstoffverbrauchs erreicht wird und auch bis das Tier von neuem ovuliert.

In Abb. 6 oben gibt die Ordinate das Zeitintervall zwischen Operation und neuer Ovulation (durch einen Punkt dargestellt) an. Die Abszisse zeigt das Trächtigkeitsalter zur Zeit der Operation. Die Zeit bis zur neuen Ovulation wird mit zunehmendem Alter verkürzt, das heisst, je näher die Operation zeitlich

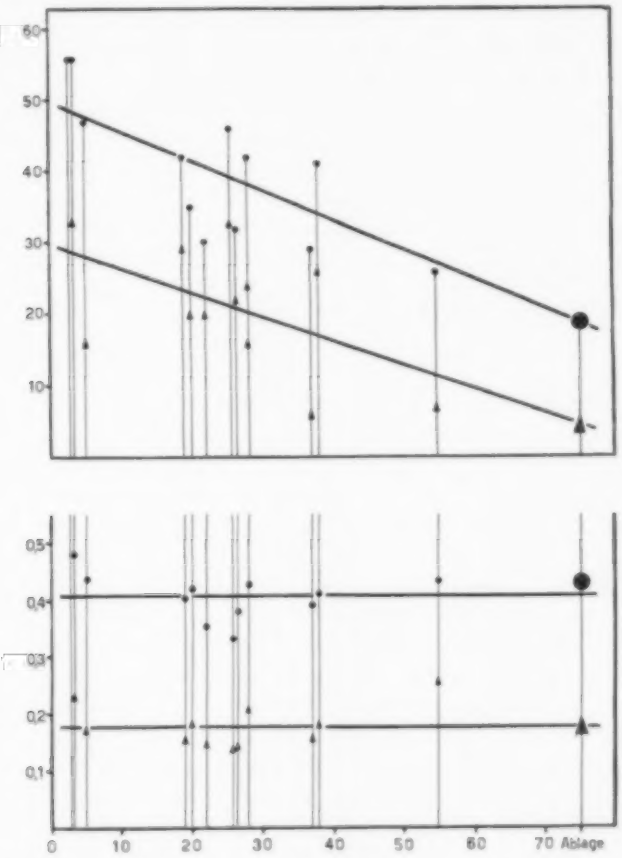

ABB. 6. Oben: Ordinate: Zeitintervall von der Entfernung des Eipakets bis zur neuen Ovulation (Punkte), bzw. bis zum O_2-Maximum (Dreiecke), in Tagen ausgedrückt. Unten: Ordinate: Sauerstoffverbrauch der entsprechenden Tiere in ml $g^{-1}h^{-1}$. Die Punkte bezeichnen das O_2-Maximum, die Dreiecke die Differenz zwischen O_2-Maximum und Verbrauch vor der Operation. Die Abszisse zeigt in beiden Abbildungen das Alter der Versuchstiere im Zeitpunkt der Operation, von der vorangehenden Ovulation an gemessen. Die grossen Punkte und Dreiecke stellen die Werte nach der Ablage dar. Bei einem Tier konnte das O_2-Maximum nicht eindeutig festgestellt werden.

an die 'normale Entfernung' des Eipakets (Ablage) geschoben wird. Die eingezeichnete Gerade ist die Verbindung des Mittelwerts des Punktschwarms mit dem (als dicken Punkt dargestellten) Wert von durchschnittlich 19 Tagen nach

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der Ablage. Rechnet man ohne diesen Wert die Regressionsgerade der 12 Punkte, so fällt sie praktisch mit der eingezeichneten theoretischen Geraden zusammen.

Die Dreiecke bezeichnen den Zeitpunkt des maximalen 0_2-Verbrauchs, das grosse Dreieck entsprechend das O_2-Maximum im Normalzyklus. Die Gerade ist wiederum die Verbindung des Schwerpunktes mit diesem Wert nach der Ablage. Hier weicht nun die Regressionsgerade etwas von der eingezeichneten ab, der errechnete P-Wert von 68% zeigt aber, dass kein Verdacht besteht, dass die Geraden nicht identisch sein könnten.

Die Prüfung der beiden Regressionen hinsichtlich Parallelität liefert einen P-Wert von ca. 55%. Es steht also nichts der Annahme entgegen, dass die durch den Mittelpunkt der beiden Punktschwärme und durch die Durchschnittswerte nach der Ablage gehenden Geraden von den beiden Regressionsgeraden nicht abweichen, und dass sie parallel sind. Für die vorliegenden Ergebnisse bedeutet das, dass bei Entfernung des Eipakets mit zunehmendem Trächtigkeitsalter der Versuchstiere sowohl das O_2-Maximum als auch die neue Ovulation immer rascher eintritt bis die normalen Zeiten nach der Ablage erreicht sind. Die Parallelität der Geraden zeigt, dass die Zeitspanne zwischen O_2-Maximum und neuer Ovulation konstant ist.

Dieses Ergebnis erlaubt nun die Interpretation der in Abb. 6 unten dargestellten Werte des maximalen Sauerstoffverbrauchs (als Punkte gezeichnet): Der O_2-Verbrauch ist nicht kleiner als der normale Maximalwert nach der Ablage. Der durchschnittliche Maximalverbrauch ist als Gerade eingezeichnet; er beträgt $0{,}406 \pm 0{,}014$ ml O_2 $g^{-1}h^{-1}$. Der Durchschnitt der Maximalwerte am 4. Tag nach der Ablage beträgt 0,425 ml O_2 und liegt somit noch innerhalb der doppelten Streuung. Die Dreiecke bezeichnen die Differenz zwischen O_2-Maximum und O_2-Verbrauch vor der Operation, also den effektiven O_2-Zuwachs. Die Gerade durch den Durchschnitt von $0{,}181 \pm 0{,}012$ ml O_2 erfasst den entsprechenden Wert nach der Ablage (0,178 ml O_2) innerhalb der einfachen Streuung. Es darf somit als erwiesen gelten, dass als Folge der experimentellen Entnahme des Eipakets der maximale O_2-Verbrauch auf den gleichen Wert steigt wie 4 Tage nach der Ablage. Es braucht aber eine umso längere Anlaufphase, je früher die Hemmung durch den Eidotter aufgehoben wird. Das Tier zeigt also im Laufe der Trächtigkeitsphase eine zunehmende Reaktionsbereitschaft. Wenn wir bedenken, dass wahrscheinlich auch hier der maximale O_2-Verbrauch nicht mit dem grössten Volumen der C. allata zusammenfällt, sondern mit der Periode des grössten Volumenzuwachses, dann wird verständlich, dass ENGELMANN für das C. allata-Volumen relativ niedrige Werte erhielt, da dieses zur Zeit der Untersuchung (15–17 Tage nach der Operation) noch nicht das Maximum erreicht haben konnte, weil die Tiere bei der Operation ein Alter von 24 bis 36 Tagen nach der Ovulation aufwiesen. (Für dieses Alter tritt der maximale O_2-Verbrauch ca. 20 Tage nach der Operation auf.)

Die O_2-Messungen nach Entfernung des Eipakets ergeben ebenfalls eine Korrelation zwischen O_2-Verbrauch und C. allata-Volumen. Dadurch werden die Befunde während des Normalzyklus gestützt. Es bleibt aber noch abzuklären, ob

der O_2-Verbrauch durch die C. allata direkt beeinflusst wird, oder ob er infolge der Eireifung ansteigt. Zur Prüfung dieser Frage wurden Kastrationsversuche durchgeführt.

b. Die Wirkung der Kastration

THOMSEN (1949) zeigte, dass die Exstirpation der C. allata bei *Calliphora*-Weibchen den O_2-Verbrauch um 24% erniedrigt. Da aber gleichzeitig eine Hemmung der Ovarien eintrat, könnte der herabgesetzte Stoffwechsel auch direkt durch das Ovar verursacht worden sein. Daher wurden kastrierte Weibchen gemessen (THOMSEN und HAMBURGER, 1955): die operierten Weibchen brauchten gleichviel Sauerstoff wie die Kontrollen, das Ovar hatte also keinen Einfluss auf

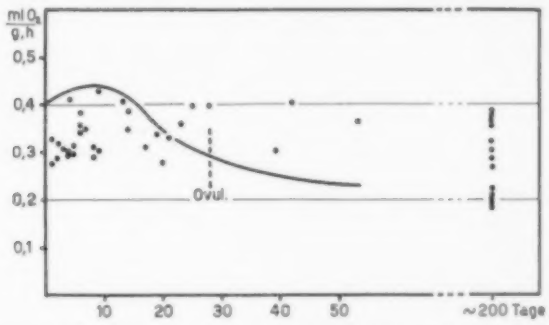

ABB. 7. Sauerstoffverbrauch von im letzten Larvenstadium kastrierten Weibchen während der ersten 53 Tage nach der Adulthäutung sowie nach ca. 200 Tagen, durch Punkte dargestellt. Die eingezeichnete Kurve zeigt den Verbrauch normaler Weibchen (vgl. Abb. 2).

den O_2-Verbrauch. Gleichzeitig wurde jedoch eine Vergrösserung der C. allata festgestellt. Da aber der O_2-Verbrauch nicht anstieg, betrachtet THOMSEN die Vergrösserung der C. allata nicht als Anzeichen erhöhter Aktivität, sondern als Hypertrophie infolge von Hormonspeicherung.

Die Frage der Bedeutung des Ovars für den O_2-Verbrauch wurde nun auch bei *Leucophaea* geprüft. Es wurden 10 weibliche Larven des letzten Stadiums kastriert; dabei blieben die medianen Teile des Geschlechtsapparates, insbesondere die Anhangsdrüsen (ENGELMANN, 1957b) in den Tieren.

Die Messungen des O_2-Verbrauchs erfolgten nach der Adulthäutung, welche 3 bis 25 Tage nach der Operation stattfand. Pro Tier wurden 2 bis 7 Messungen im Alter von 1 bis 53 Tagen nach der Häutung durchgeführt. Innerhalb dieser Zeit blieb der O_2-Verbrauch konstant (Abb. 7). Der Durchschnitt der Mittelwerte für jedes Tier lieferte den Gesamtdurchschnitt von $0{,}342 \pm 0{,}011$ ml O_2 $g^{-1}h^{-1}$.

Im Alter von rund 200 Tagen nach Adulthäutung wurden 13 Weibchen gemessen.* Aus den Messungen ergab sich ein Mittel von $0{,}268 \pm 0{,}023$ ml O_2, dh., es lässt sich kein Unterschied sichern zum Trächtigkeitswert von 0,247 ml O_2.

* In 6 dieser Weibchen waren nach dem 50. Tag C. allata implantiert worden, was jedoch das hier angeführte Resultat nicht beeinflusst hat.

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Kastrierte Weibchen benötigen also in den ersten 53 Tagen nach der Imaginalhäutung mehr Sauerstoff als 200 Tage nach der Häutung. Die Differenz ist allerdings nicht gesichert ($P = 0{,}05$).

c. Implantation von Corpora allata in kastrierte Weibchen

Kastrierte Weibchen sind als Testobjekte für Implantationsversuche besonders geeignet, da keine Eireifung ausgelöst wird. Es kann somit entschieden werden, ob das Ovar eine Wirkung auf den respiratorischen Stoffwechsel ausübt oder nicht. Es besteht allerdings noch die Möglichkeit, dass die Aktivierung der Anhangsdrüsen einen erhöhten O_2-Verbrauch zur Folge hat. Je 4 larval kastrierten,

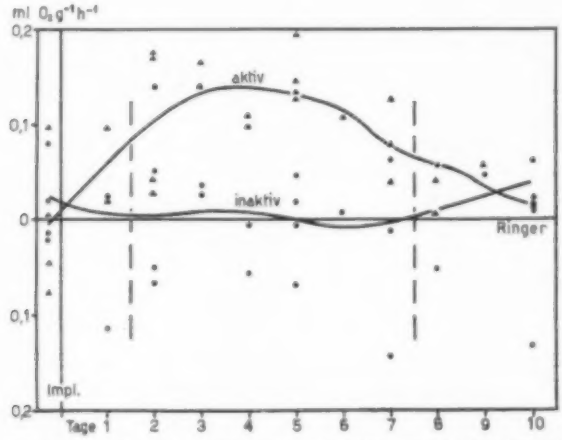

ABB. 8. Sauerstoffverbrauch kastrierter Weibchen nach Implantation von aktiven C. allata (Dreiecke) und inaktiven C. allata (Punkte), als Differenz zur Ringerkontrolle dargestellt.

adulten Weibchen wurden 1 Paar aktive C. allata, inaktive C. allata oder Ringerlösung in das Abdomen injiziert. Der O_2-Verbrauch der Spender und Wirte wurde vor der Operation gemessen. Die aktiven C. allata entstammten Weibchen, welche 4 Tage vorher abgelegt hatten (die Drüsen sind also hinsichtlich des Sauerstoffverbrauchs 'aktiv', in Bezug auf das Volumen sind sie erst im Wachstum begriffen), die inaktiven aus trächtigen Weibchen.

Abb. 8 zeigt die Ergebnisse dieses Versuchs. Da 6 Tiere einige Tage, die andern 6 jedoch erst 200 Tage nach der Adulthäutung operiert wurden, ist der Verbrauch vor der Operation verschieden (vgl. Abb. 7). Daher wurden nicht die absoluten O_2-Werte für die Darstellung verwendet, sondern die Differenz zur Ringerkontrolle, welche als Waagrechte eingezeichnet ist. Die Höhe der Dreiecke bezeichnet den Mehrverbrauch der Tiere mit aktiven Implantaten gegenüber dem jeweiligen Kontrollwert, die Punkte bezeichnen entsprechend den Verbrauch der Tiere mit inaktiven Implantaten. Zu jedem Dreieck gehört ein Punkt und eine Ringerkontrolle. Die drei zusammengehörigen Tiere sind gleich alt und

wurden gleichzeitig larval kastriert; sie erhielten auch am gleichen Tag ein Implantat.

Der Sauerstoffverbrauch der Tiere mit aktiven Implantaten steigt nach der Operation an und erreicht am 3. bis 5. Tag ein Maximum ($0{,}117 \pm 0{,}02$ ml O_2 $g^{-1}h^{-1}$ mehr als der Ringerwert), am 9. Tag fällt er auf das Niveau der Kontrolltiere zurück. Inaktive Drüsen hatten keine Wirkung. Innerhalb der willkürlich gewählten Grenzen (2. und 7. Tag, gestrichelte Ordinaten) ergeben die Ringerkontrollen einen Durchschnitt von $0{,}326 \pm 0{,}018$ ml O_2 $g^{-1}h^{-1}$, der in Abb. 8 durch die Waagrechte auf der Höhe Null dargestellt ist (also: $0{,}00 \pm 0{,}018$). Der Durchschnitt der Differenzen des Sauerstoffverbrauchs der Tiere mit inaktiven

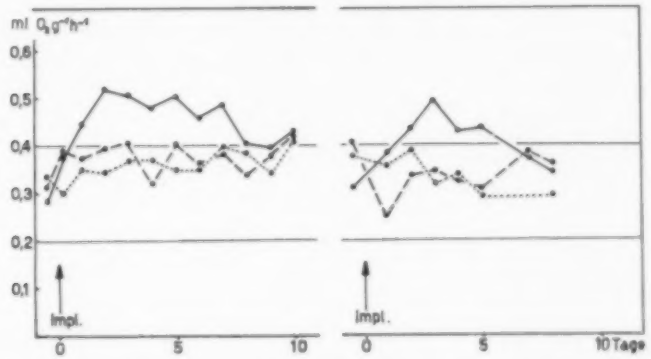

ABB. 9. Sauerstoffverbrauch kastrierter Weibchen nach C. allata-Implantation. Einzeldarstellung zweier Versuche. —— Implantation aktiver C. allata, – – – – – Implantation inaktiver C. allata, ... Ringerkontrolle.

Implantaten zu den Ringerkontrollen beträgt $0{,}003 \pm 0{,}033$ ml O_2. Der Unterschied der Wirkung aktiver und inaktiver Implantate ist mit $P = 0{,}02$ gesichert. Es zeigt sich, dass das Volumen an sich kein Mass für die Wirkung ergibt, denn die inaktiven Implantate haben anscheinend gar kein Stoffwechselhormon geliefert. Wahrscheinlich stellt also der Sauerstoffverbrauch trächtiger Weibchen (0,247 ml O_2) den von den C. allata unabhängigen 'Grundumsatz' dar. Es wurde denn auch kein tieferer Durchschnittsverbrauch festgestellt.

Da Abb. 8 die Differenzen des Sauerstoffverbrauchs darstellt, ist es nicht möglich, den wahren Verlauf der Kurven und insbesondere den Zusammenhang der Werte für einzelne Tiere zu erkennen. Abb. 9 zeigt die Einzelkurven zweier Versuchsserien: Der Verbrauch der Tiere mit aktiven Implantaten ist höher als derjenige der Tiere mit inaktiven Implantaten oder der Ringerkontrollen, welche sich oft überschneiden. Es ist zu beachten, dass für die Implantation aktiver C. allata immer das Tier benützt wurde, das vor der Operation den geringsten Verbrauch aufgewiesen hatte.

Die Implantation inaktiver C. allata erhöht den Sauerstoffverbrauch gegenüber den Ringerkontrollen nicht, werden aber aktive Drüsen implantiert, dann wird der

respiratorische Stoffwechsel vorübergehend gesteigert. Hier muss also eine direkte Wirkung der C. allata auf den O_2-Verbrauch vorliegen. Dass dieser nach einigen Tagen wieder zurückgeht, liegt wohl an einer Inaktivierung der Implantate. Auch bei *Dixippus* (PFLUGFELDER, 1952) wird der O_2-Verbrauch nur vorübergehend—während ca. 20 Tagen—durch Implantation aktiver C. allata gesteigert.

d. Implantation von Corpora allata in Weibchen, deren gesamter Geschlechtsapparat entfernt wurde

Ausser der Auslösung der Eireifung bewirken die C. allata während jeder Eireifungsphase eine Aktivierung der Anhangsdrüsen (ENGELMANN, 1957b), welche des Sekret für die Bildung des Kokons liefern. Die Anhangsdrüsen benötigen wahrscheinlich zur Sekretbildung ebenfalls Energie. Sie könnten daher auch bei den Kastrationsversuchen den respiratorischen Stoffwechsel beeinflussen. Um diese Möglichkeit zu prüfen, wurde bei 16 Weibchen der gesamte Geschlechtsapparat exstirpiert.

Der Durchschnitt des O_2-Verbrauchs der Weibchen ohne Geschlechtsapparat beträgt $0{,}333 \pm 0{,}019$ ml O_2 $g^{-1}h^{-1}$ und ist nicht verschieden vom Verbrauch der kastrierten Weibchen.

Die operierten Weibchen wurden wie folgt verwendet: 5 Tieren wurden aktive C. allata, 5 Tieren inaktive C. allata implantiert; 6 Tieren wurde Ringerlösung injiziert. Gemessen wurde der Sauerstoffverbrauch unmittelbar vor, sowie 4 und 5 Tage nach der Operation, da nach dem Versuch mit kastrierten Weibchen in dieser Zeit das Maximum des Verbrauchs erwartet wurde. Der Vergleich der Sauerstoff-Verbrauchsdifferenzen zwischen dem Verbrauch vor der Operation und dem Mittel aus den beiden Messungen nach der Operation ergibt folgendes Bild:

Der Verbrauch der Weibchen mit aktiven Implantaten erhöhte sich um $0{,}107 \pm 0{,}013$ ml O_2 $g^{-1}h^{-1}$. Der Verbrauch der Weibchen mit inaktiven Implantaten erhöhte sich um $0{,}001 \pm 0{,}029$ ml O_2, blieb also gleich wie vor dem Eingriff.

Der Verbrauch der Ringerkontrollen sank um $0{,}012 \pm 0{,}019$ ml O_2, blieb also ebenfalls praktisch gleich.

Der Sauerstoff-Verbrauch der Weibchen mit aktiven Implantaten ist mit einem $P = 0{,}01$ von denjenigen mit inaktiven Implantaten und Ringerkontrollen gesichert verschieden.

Der Verbrauch der Weibchen mit inaktiven Implantaten dürfte demjenigen der Ringerkontrollen entsprechen ($P = 0{,}76$). Das Ergebnis ist also das gleiche wie nach der Operation kastrierter Weibchen. Die Erhöhung des O_2-Verbrauchs ist somit nicht nur eine Folge der Eireifung und der Aktivität der Anhangsdrüsen.

V. DER SAUERSTOFFVERBRAUCH DER LARVEN

Im vorangehenden Abschnitt konnte gezeigt werden, dass der Sauerstoffverbrauch bei adulten Weibchen durch ein Hormon der C. allata beeinflusst wird, und dass dieses den respiratorischen Stoffwechsel beeinflussende Hormon möglicherweise mit dem Eireifungshormon identisch ist. In den Larvenstadien

geben die C. allata das Juvenilhormon ab. Da es bis heute nicht gelungen ist, abzuklären, ob das Juvenilhormon mit dem Eireifungshormon identisch ist, war es von Interesse zu untersuchen, ob auch in den Larvenstadien der Sauerstoffverbrauch mit der Aktivität der C. allata korreliert ist.

Bei *Leucophaea* werden unter normalen Zuchtbedingungen 8 Larvenstadien durchgemacht (ENGELMANN, 1957b). In den ersten 7 Larvenstadien produzieren die C. allata Juvenilhormon. Im letzten Stadium dagegen werden sie inaktiviert (SCHARRER, 1946; LÜSCHER und ENGELMANN, 1960). Es war deshalb zu erwarten, dass bei Identität der Hormone die O_2-Verbrauchskurve im letzten Stadium von derjenigen der früheren Larvenstadien wesentlich abweichen sollte. Der O_2-Verbrauch wurde im zweitletzten und im letzten Larvenstadium gemessen (Abb. 10).

a. Das zweitletzte Larvenstadium

5 weibliche Larven wurden in Abständen von 2 bis 4 Tagen gemessen. Die Werte wurden auf einen Durchschnitt von 34,2 Tage ausgeglichen (ENGELMANN, 1957b: 26,8 Tage). Abb. 10 zeigt, dass nach der Häutung ein Anstieg auf 0,425 ml $O_2\ g^{-1}h^{-1}$ erfolgt (dieser Wert entspricht dem nach der Ablage und bei Eipaketentfernung erreichten Maximalwert). Daraufhin fällt der O_2-Verbrauch auf ca. 0,2 ml, um dann gegen Ende des Stadiums wieder anzusteigen. Das Volumen der C. allata ist charakterisiert durch ein Maximum am Anfang, durch eine Volumenabnahme und einen neuerlichen Anstieg von der Mitte des Stadiums an (LÜSCHER und ENGELMANN, 1960). Die Maximalwerte des O_2-Verbrauchs und des C. allata-Volumens nach der Häutung fallen zeitlich zusammen, die einsetzende Produktion von Juvenilhormon, welches nach SCHARRER (1946) 16 Tage vor der nächsten Häutung ihre kritische Phase hat (für die vorliegenden Durchschnittswerte also am 18. Tag), bewirkt aber keine Steigerung des Stoffwechsels.

b. Das letzte Larvenstadium

Die gleichen 5 Larven wurden auch während des letzten Larvenstadiums alle 2 bis 4 Tage gemessen. Dazu wurden noch einige weitere Einzelmessungen verwertet (Abb. 10). Der Verbrauch an O_2 folgt ungefähr den gleichen Gesetzmässigkeiten wie im 7. Stadium, allerdings ist das erste Maximum etwas später und weniger hoch, das Minimum etwas weniger tief, und das Maximum gegen Ende des Stadiums wird ebenfalls später erreicht, liegt aber deutlich höher. Das Volumen der C. allata (LÜSCHER und ENGELMANN, 1960) zeigt die gleichen Veränderungen wie im zweitletzten Stadium, nur ist der Anstieg vor der Häutung hier auf eine Zellvermehrung zurückzuführen und nicht auf eine Zellvergrösserung. In beiden Stadien erkennt man nur nach der Häutung einen Zusammenhang zwischen C. allata-Volumen und O_2-Verbrauch. Im übrigen besteht aber keine Korrelation zur Juvenilhormonaktivität: die beiden Kurven unterscheiden sich nur wenig voneinander. Weder die Aktivität in der Mitte des 7. Stadiums, noch die Inaktivierung im letzten Stadium (LÜSCHER und ENGELMANN, 1960) lassen sich an dem Verlauf der Kurven ablesen. Die larvalen C. allata scheinen also

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keine stoffwechselstimulierende Wirkung zu haben, und das Juvenilhormon ist wahrscheinlich nicht identisch mit dem stoffwechselwirksamen Hormon der C. allata. ZWICKY und WIGGLESWORTH (1956) konnten zeigen, dass der Verlauf des O_2-Verbrauchs der Larven der blutsaugenden Wanze *Rhodnius* in jedem Stadium ein ähnliches Bild ergibt: nach der Fütterung mit Blut, wodurch die nächste Häutung ausgelöst wird, steigt der O_2-Verbrauch zuerst stark, dann schwächer an, er erreicht nach neuerlichem Anstieg das Maximum während der

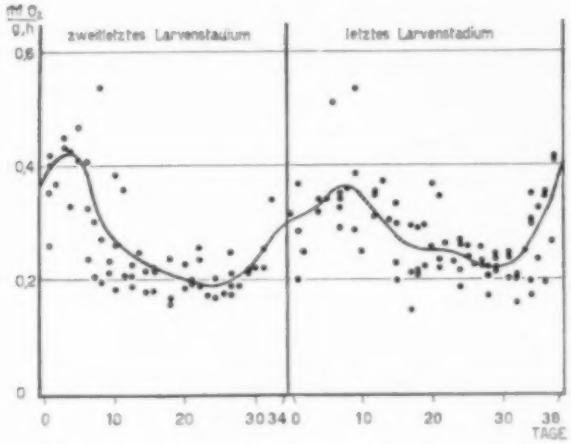

ABB. 10. Sauerstoffverbrauch der Larven des zweitletzten und letzten Stadiums. Abszisse: Dauer der Stadien in Tagen.

Häutung und fällt dann rasch auf das Minimum zurück. Der Imaginalhäutung geht derselbe Anstieg voraus wie den Larvenhäutungen, wobei der Verlauf der Kurve durch die einmalige Blutmahlzeit in jedem Stadium bestimmt wird. Die Autoren zeigen unter Bezugnahme auf die Histologie der Häutung sowie durch Hemmung des Cytochromsystems, dass der O_2-Verbrauch durch die Proteinsynthese bedingt ist. Der zunehmende O_2-Verbrauch vor der Häutung kann bei *Leucophaea* ebenfalls durch die Proteinsynthese erklärt werden, wobei der nach der Häutung fortdauernde Anstieg durch die Ablagerung der Endocuticula sowie durch die Aktivität der Polyphenoloxydasen in der Cuticula verursacht sein dürfte.

VI. DISKUSSION

a. Der Nachweis der Stoffwechselwirkung der Corpora allata

Die Versuche mit normalen, adulten Weibchen und Männchen zeigen, dass zwischen dem Volumen der C. allata und dem Stoffwechsel (gemessen durch den Sauerstoffverbrauch) folgender Zusammenhang besteht: Der Sauerstoffverbrauch erreicht dann ein Maximum, wenn die C. allata am stärksten anwachsen; die

Kurve des Sauerstoffverbrauchs läuft also der Volumenkurve der C. allata vor (s. Abb. 2).

Die experimentelle Entfernung des Eipaketes bewirkt eine Vergrösserung des C. allata-Volumens (LÜSCHER und ENGELMANN, 1955) und eine Steigerung des Sauerstoffverbrauchs, wobei dieselben Maximalwerte erreicht werden wie nach der normalen Ablage.

Auch bei kastrierten Weibchen scheint eine Beziehung zwischen C. allata-Volumen und respiratorischem Stoffwechsel zu bestehen. Der O_2-Verbrauch liegt zwischen Maximal- und Minimalverbrauch normaler Weibchen. Eine rasche Volumenzunahme der C. allata nach Kastration wurde bei *Calliphora* (THOMSEN und HAMBURGER, 1955), bei *Melanoplus* (PFEIFFER, 1945), bei *Lucilia* und *Sarcophaga* (DAY, 1943) uam. beobachtet. Bei *Leucophaea* allerdings soll eine Volumenzunahme erst vom 4. bis 8. Monat nach der Imaginalhäutung erfolgen (VON HARNACK und SCHARRER, 1956). In diesem Falle ist aber aus der betreffenden Arbeit nicht ersichtlich, auf welches Ausgangsvolumen Bezug genommen wurde.

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Die Implantation aktiver (für den O_2-Verbrauch) C. allata aus Weibchen, welche 4 Tage vorher abgelegt hatten, in kastrierte Weibchen und in solche, deren ganzer Geschlechtsapparat entfernt wurde, bewirkt eine starke, allerdings nur einige Tage dauernde Steigerung des O_2-Verbrauchs. Die Implantation inaktiver C. allata aus trächtigen Weibchen hat keine Wirkung. Die Funktionen der normalerweise unter dem Einfluss der C. allata stehenden Geschlechtsorgane, speziell Ovar und Anhangsdrüsen, können also für den Mehrverbrauch nicht allein verantwortlich sein. Damit ist der Nachweis der direkten stoffwechselstimulierenden Wirkung der C. allata erbracht.

Ein ähnliches Ergebnis erzielte auch PFLUGFELDER (1952) bei *Dixippus*, doch waren die Wirte nicht kastriert, weshalb der Autor den vorübergehend erhöhten O_2-Verbrauch der Tätigkeit des Ovars zuschreibt. NOVÁK und Mitarbeiter (1959) fanden keine Wirkung nach Implantation von C. allata in kastrierte Weibchen von *Pyrrhocoris apterus*. Es wurden jedoch Hungertiere verwendet, die wohl keine abbaufähigen Fettreserven hatten (s. unten). THOMSEN (1949) konnte bei *Calliphora*-Männchen nach Implantation von C. allata ebenfalls keine Steigerung des Stoffwechsels beobachten (es besteht die Möglichkeit, dass inaktive C. allata verwendet wurden), wohl aber nach Implantation von drei Drüsen in *Calliphora*-Weibchen, wobei der O_2-Verbrauch um 19% gesteigert wurde.

b. Der Angriffspunkt des Corpora allata-Hormons im Stoffwechsel

Vorläufig sind wir nur auf Vermutungen angewiesen über den Ort des Eingreifens des C. allata-Hormons in den Stoffwechsel. Die Beeinflussung einzelner Stoffwechselvorgänge, wie sie insbesondere durch L'HÉLIAS (1957) bei *Dixippus* untersucht worden sind, kann sowohl als Angriff an verschiedenen Punkten wie auch als Folge einer einzigen Stimulierung erklärt werden, welche sich notwendigerweise auch in einem erhöhten Sauerstoffverbrauch äussern muss.

Bei vielen Insekten bewirkt die Exstirpation der C. allata eine Vergrösserung des Fettkörpers, so bei *Drosophila* (VOGT, 1947). *Calliphora* (THOMSEN, 1942) und bei der Heuschrecke *Melanoplus differentialis* (PFEIFFER, 1945), bei welcher auch eine Zunahme der Fettsäuren nachgewiesen wurde. Bei *Sarcophaga* und *Lucilia* (DAY, 1943) persistieren nach Allatektomie die larvalen Fettzellen in der Imago. Die C. allata stimulieren also den Fettabbau. Es ist zu vermuten, dass ein für den oxydativen Abbau der Fettsäuren notwendiges Atmungsenzym gefördert wird. Für eine direkte Wirkung auf ein Enzymsystem spricht auch der Befund von DE WILDE und STEGWEE (1958) an Diapausetieren von *Leptinotarsa*, dass C. allata nicht nur in vivo, sondern auch in vitro in Gewebehomogenaten den O_2-Verbrauch erhöhen.

Bei Kastration bleibt der Fettgehalt trotz erhöhter C. allata-Aktivität und gesteigertem O_2-Verbrauch fast unverändert (PFEIFFER, 1945). Es wird wohl in diesem Falle so viel Nahrung aufgenommen, dass das verbrauchte Fett immer wieder ersetzt wird. Qualitative Untersuchungen an *Calliphora*-Weibchen (THOMSEN und HAMBURGER, 1955) zeigen, dass durch Kastration der Fettgehalt nur gegenüber Weibchen mit reifen Eiern gesteigert wird, nicht aber gegenüber solchen mit Eiern verschiedener Entwicklungsstadien. Es konnte auch ein erhöhter Glykogengehalt nachgewiesen werden. Die Frage, wie die entstehende Energie, die bei kastrierten Wiebchen nicht zur Proteinsynthese herangezogen wird, verwendet wird, bleibt noch offen. Möglicherweise bewirkt sie wie das Thyroxin bei Säugern erhöhte Temperatur und gesteigerte Aktivität.

c. Die Beziehung zwischen Volumen und Aktivität der Corpora allata

Kleine C. allata aus trächtigen Weibchen haben bei der Implantation in kastrierte Weibchen keine Wirkung. In Bezug auf die Stimulierung des respiratorischen Stoffwechsels sind sie vollkommen inaktiv. Das Volumen an sich ist also kein zuverlässiges Mass für die Aktivität. Für die Wirkung des Stoffwechselhormons ergibt sich Folgendes: Kleine Drüsen sind inaktiv, grosse Drüsen sind manchmal aktiv (bei kastrierten Weibchen), aber nicht immer (wenn während der Eireifungsperiode das grösste Drüsenvolumen erreicht wird, ist der O_2-Verbrauch wieder am Abnehmen). Die grösste Aktivität haben die C. allata zur Zeit ihres stärksten Wachstums (Cytoplasmazunahme).

d. Die Identität der verschiedenen Corpora allata-Hormone

Die Korrelation zwischen O_2-Verbrauch und C. allata-Volumen weist darauf hin, dass das Eireifungshormon und das den respiratorischen Stoffwechsel stimulierende Hormon möglicherweise identisch sind. Die mannigfaltigen Wirkungen nach Exstirpation der C. allata (Degeneration ausdifferenzierter Gewebe (PFLUGFELDER, 1938), Wucherungen (PFLUGFELDER, 1938), Regenerationshemmung (PFLUGFELDER, 1939) könnten eine Folge des herabgesetzten Stoffwechsels sein. Die Eireifung selbst könnte auf der Stoffwechselwirkung beruhen, bzw. durch sie ermöglicht werden.

Die Tatsache, dass Sauerstoffverbrauch im zweitletzten und im letzten Larvenstadium dieselben Gesetzmässigkeiten aufweist, legt die Vermutung nahe, dass es sich bei Stoffwechsel- und Juvenilhormon um zwei verschiedene Stoffe handelt, deren Ausschüttung durch das larvale oder imaginale Milieu gesteuert wird. Das Milieu könnte jedoch auch die Wirkung eines einzigen Hormons beeinflussen, das dann in der Larve als Juvenilhormon, im adulten Tier dagegen stoffwechselstimulierend wirken würde, doch scheint uns dies wenig wahrscheinlich.

Die vorliegenden Untersuchungen machen eine Identität des Stoffwechselhormons mit dem Eireifungshormon wahrscheinlich. Stoffwechselhormon und Juvenilhormon dagegen scheinen nicht identisch zu sein. Die Tatsache, dass adulte C. allata, wenn sie in Larven implantiert werden, juvenil wirken (bei *Dixippus*, PFLUGFELDER, 1940), und dass juvenile C. allata in adulten Weibchen von *Rhodnius* die Eireifung stimulieren (WIGGLESWORTH, 1936) spricht nicht gegen die Zweihormontheorie, da die Art der Ausschüttung vom Milieu bestimmt sein könnte. Eine Klärung dieser Frage der Identität der Hormone wird nur möglich sein, wenn einmal das Stoffwechselhormon oder das Juvenilhormon in reiner Form für Injektionsversuche zur Verfügung steht.

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VII. ZUSAMMENFASSUNG

1. Der Sauerstoffverbrauch von Larven und adulten Weibchen und Männchen der ovoviviparen Schabe *Leucophaea maderae* wurde mit Hilfe eines speziellen Umlaufrespirometers geprüft.

2. Adulte Weibchen weisen einen mit der Eireifung und der Trächtigkeit zusammenhängenden Zyklus der Corpora allata-Aktivität auf. Der Sauerstoffverbrauch ist mit diesem korreliert. Das Maximum des Sauerstoffverbrauchs tritt jeweils dann auf, wenn die Volumenzunahme der Corpora allata am stärksten ist.

3. Bei adulten Männchen weisen sowohl das Volumen der Corpora allata als auch der Sauerstoffverbrauch ein vorübergehendes Maximum auf und bleiben dann konstant. Auch hier tritt das Sauerstoffmaximum früher auf als das Volumenmaximum.

4. Durch Entfernen des Eipaketes bei trächtigen Weibchen wird die Hemmung der Corpora allata aufgehoben und deren Volumen steigt an. Der Sauerstoffverbrauch nimmt ebenfalls zu. Das Maximum wird umso früher erreicht, je später die Operation durchgeführt wird, wobei sich auch die Zeit bis zur nächsten Ovulation verkürzt. Die Zeit zwischen dem Maximum des Sauerstoffverbrauchs und der neuen Ovulation ist konstant.

5. Der Sauerstoffverbrauch von Weibchen, die im letzten Larvenstadium kastriert wurden, bleibt nach der Adulthäutung längere Zeit erhöht und sinkt dann auf den Inaktivitätswert.

6. Implantationen aktiver Corpora allata in kastrierte Weibchen und in solche, deren Geschlechtsapparat ganz fehlt, erhöht für kürzere Zeit den Sauerstoffverbrauch, während inaktive Corpora allata und Ringerlösung keine Wirkung haben. Damit ist die direkte, stoffwechselstimulierende Wirkung des Corpora allata-Hormons in adulten Weibchen erwiesen.

7. Der Sauerstoffverbrauch im zweitletzten und letzten Larvenstadium weist dieselben Gesetzmässigkeiten auf, obschon im letzten Larvenstadium kein Juvenilhormon ausgeschüttet wird.

8. Das Hormon, welches den respiratorischen Stoffwechsel stimuliert, ist wahrscheinlich identisch mit dem gonadotropen Hormon, nicht aber mit dem Juvenilhormon.

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CRYPTOBIOSIS IN THE LARVA OF *POLYPEDILUM VANDERPLANKI* HINT. (CHIRONOMIDAE)

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Abstract—Metabolism in the larva of *Polypedilum vanderplanki* can be brought reversibly to a standstill by dehydration and exposure to very low or very high temperatures. Larvae metamorphosed after immersion in liquid air or liquid helium when dry. Some metamorphosed after exposure to 102–104°C for 1 min when dry, and some recovered temporarily after exposure to 106°C for 3 hr or 200°C for 5 min. Larvae recovered after immersion in absolute ethanol or pure glycerol when dry. One larva metamorphosed after immersion in absolute ethanol for 24 hr, and all larvae recovered temporarily after immersion for 7 days. Larvae immersed in glycerol for 1, 3, and 7 days respectively metamorphosed. Some larvae recovered temporarily after being stored dry for 10 years. They tolerate repeated dehydration: one larva metamorphosed after ten dehydrations to a moisture content of about 8 per cent or less, each separated by several days in water during which it fed.

INTRODUCTION

THE Chironomid, *Polypedilum vanderplanki* Hint., breeds in small pools formed in shallow hollows on unshaded rocks in Northern Nigeria and Uganda. Its larva is especially interesting because it is the only insect that is definitely known to be able to survive more or less total dehydration (HINTON, 1951). A supply of larvae recently obtained from Africa has made possible the further experiments described in this paper.

When the moisture content of dry larvae was plotted against relative humidity, it was found that at 0 per cent r.h. it was less than 3 per cent, at 60 per cent r.h. it was 8 per cent, and at higher humidities the curve rose more steeply. For instance, when larvae were transferred from 0 to 100 per cent r.h. their moisture content rose to 33 per cent within 6 hr. The figures for moisture content are based on the assumption that the larva contains no water when its weight is constant at 106°C. When dry larvae are placed in water, the pharynx and dorsal vessel begin to beat when the moisture content rises to about 55 per cent or so, but they have to take up much more water before they can crawl or before they swell to their normal shape when active.

The metabolism of the larva can be brought reversibly to a standstill by dehydration and exposure to very low or very high temperatures, as noted below. At physiological temperatures the oxygen uptake of the larva rises as its moisture content rises, as will be shown in a later paper. It has previously been shown (HINTON, 1951) that the oxygen uptake of 'dry' larvae is 0·0023 μl per larva per hr, which is 171 times less than that of active, hydrated larvae. The moisture content

of the 'dry' larvae used was not then determined, but as a 10 per cent solution of KOH was used in the Warburg, the relative humidity to which the larvae were exposed in the experiments was probably about 92 per cent, and at such a humidity the moisture content of the larva is close to 30 per cent.

It seems that if dry larvae are to recover from dehydration they must be dried slowly. For instance, of 154 larvae that were dried on glass either at room temperatures at 60 per cent r.h., or at 25°C at about 40 per cent r.h., only a few recovered and then only temporarily when subsequently placed in water. Larvae recovered from dehydration at the same temperatures and humidities when they were placed in a small quantity of wet mud or in a covered Petri dish containing damp filter paper. The impression was gained that within the range of drying speeds that ensured a recovery of nearly 100 per cent, the faster they were dried the more slowly they recovered when subsequently placed in tap water.

SURVIVAL AFTER PROLONGED DEHYDRATION

It has previously been recorded (HINTON, 1953) that larvae stored in dry mud for 3 years and 3 months at room temperatures and humidities produced normal adults when placed in tap water. Recent attempts to activate larvae that had been

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TABLE 1—RECOVERY OF LARVAE STORED AT ROOM TEMPERATURE FOR PERIODS OF 7 AND 10 YEARS RESPECTIVELY

Years dry	Relative humidity during storage (%)	Hours at 100% r.h. before immersion in water	No. of larvae	No. recovered
7	40–80	0	120	0
7	40–80	3	10	1*
10	40–80	0	108	2*
10	40–80	4	9	0
10	40–80	3	10	0
10	40–80 (7 years ca. 4%)	0	24	0
10	40–80 (7 years ca. 4%)	4	7	6
10	40–80 (7 years ca. 4%)	3	11	9
10	40–80 (7 years ca. 4%)	20–24	8	0

* Recovery doubtful.

stored at room temperatures for 7 and 10 years respectively in the dry mud in which they had been collected in the field were only partially successful: some larvae made a temporary recovery but none survived the prolonged dehydration (Table 1). Since many movements of the body may be due entirely to the mechanical effects of absorbing water rapidly, larvae were considered to have recovered after prolonged dehydration only if after immersion more or less regular pharyngeal or dorsal vessel contractions were evident, or if, in the absence of either, they responded to touch by making distinct avoiding movements. Larvae were immersed only in small groups, usually five or six, since it is very difficult to observe more than this number at the same time; and many of the entries in Table 1 are therefore the sum of a number of experiments.

As previously noted, larvae that had been dehydrated for 39 months successfully recovered when at the end of this period they were immersed directly in tap water. When a number of larvae that had been stored at room humidities for 7- and 10-year periods failed to recover in tap water, various saline solutions up to a pressure of 8 atm were tried without success. Small amounts of ATP up to 25 μg/ml were added to the tap water or to the saline, also without success. Most of the supply of 7- and 10-year old larvae had been used before it was discovered that exposure to a relative humidity of about 100 per cent for a few hours before immersion in tap water greatly enhanced recovery. Even so, the only clear instances of recovery were obtained with the material that had been stored for 3 years at room humidities and then over calcium chloride for a further 7 years. As shown in Table 1, 83 per cent of the larvae made a temporary recovery when exposed for 3 or 4 hr to 100 per cent r.h. before immersion in water. Most of the larvae that recovered temporarily after 10 years of dehydration ceased to show any signs of life after about 2 hr in water, but the dorsal vessel of one specimen was beating regularly a little more than 5 hr 30 min after immersion.

The data recorded in Table 1 suggest that the capacity of 'dry' inactive larvae to survive prolonged periods is greater the drier they are, which is what would be expected on general grounds. For instance, storage at relative humidities of 40–80 per cent for 7 years is more damaging to them than storage at the same humidities for 3 years followed without hydration by a further period of storage for 7 years at a relative humidity of 4 per cent.

TOLERANCE TO REPEATED DEHYDRATION

The capacity of larvae to tolerate repeated dehydration is clearly of selective value: during much of the year the pools in which they live may repeatedly fill and dry. It has been shown (HINTON, 1951) that larvae grow normally after having been subjected to as many as three dehydrations of 24 hr, each separated by an interval of 3 hr in water during which they began to feed. That larvae can tolerate many more than three dehydrations is evident from the results summarized in Fig. 2.

In the first experiment (Fig. 2, A–D) four dry larvae were placed in water for a few days. They were then placed on moist filter paper in a covered Petri dish in an incubator held at 25 ± 1°C and allowed to dry as the filter paper dried. After 3 days their appearance suggested a moisture content of about 15 per cent or less, and they were then placed in water and allowed to feed for 2 days. For the third and subsequent dehydrations they were placed in a glass tube at the bottom of which was a column of wet mud 5–8 mm high and 5 mm in diameter. The larvae burrowed into the mud. When the mud appeared to be dry, the tube was heated to 56–57°C for 2 hr in an electric oven. Survival after this heat treatment was considered to be certain evidence that the larvae concealed in the dry mud were dehydrated: hydrated larvae are irreversibly injured at 56°C in about 1 min, and the 3 mm of glass and dry mud will not have shielded them against this temperature for more than a small fraction of the 2 hr. After the heat treatment,

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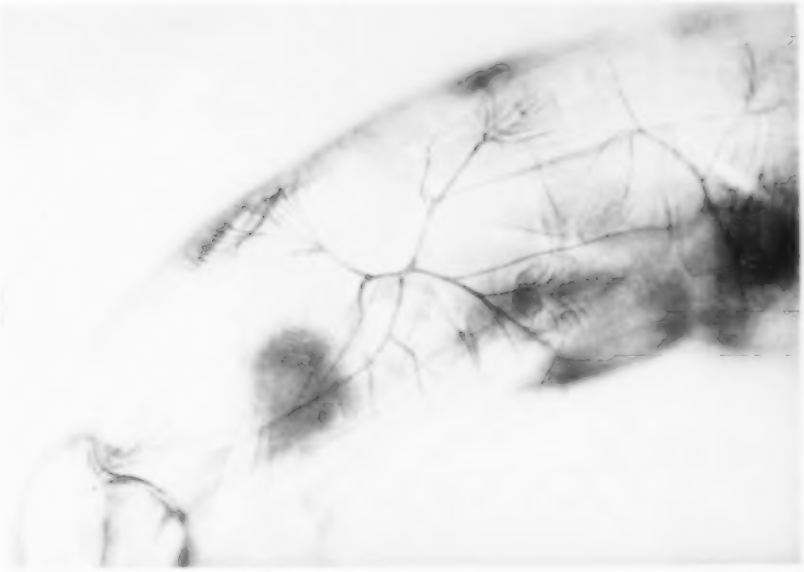
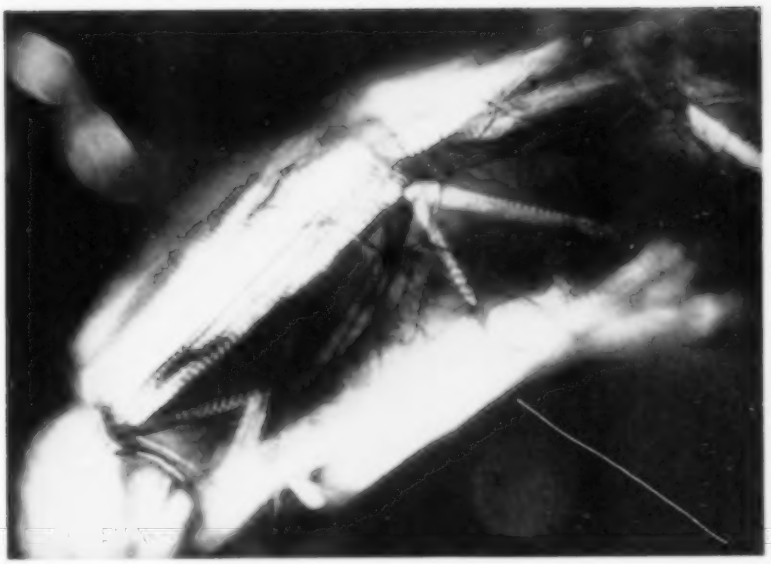
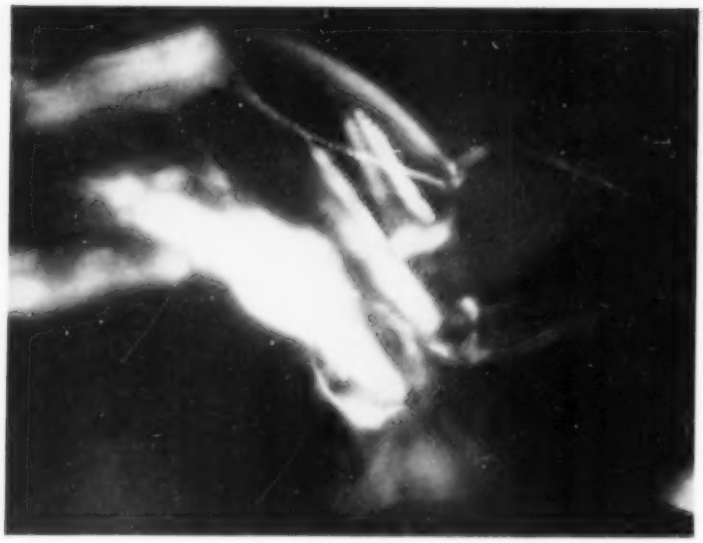


(a)



(b)

FIG. 1. Larvae of *Polypedilum vanderplanki* after immersion in tap water following a 10-year period of continuous dehydration. (a) Lateral view of thorax. (b) Lateral view of thorax.



(c)

(d)

(e)

FIG. 1 (*cont.*). (c) Lateral view of end of abdomen. (d) Middle of abdomen. (e) Middle of abdomen.

the larvae were washed out of the mud, allowed to feed in tap water in Petri dishes in an incubator at 25 ± 1°C for the times indicated, and then placed in another tube with wet mud for a further dehydration. As may be seen in Fig. 2, A–D, one larva died after the fourth and one after the seventh dehydration. One larva escaped after the fourth and another after the seventh dehydration.

In the second experiment (Fig. 2, E–H) four dry larvae were placed in water and allowed to feed for 6 days. They were then placed in wet mud in a glass tube as in the first experiment. Only one larva survived the third and fourth dehydrations, probably because these dehydrations were insufficient to protect them against the heat treatment. The cause of death of the larva that survived the fourth dehydration but died after the sixth (Fig. 2, E) is not known, but its death may possibly have resulted from heat injury after the very brief fourth dehydration.

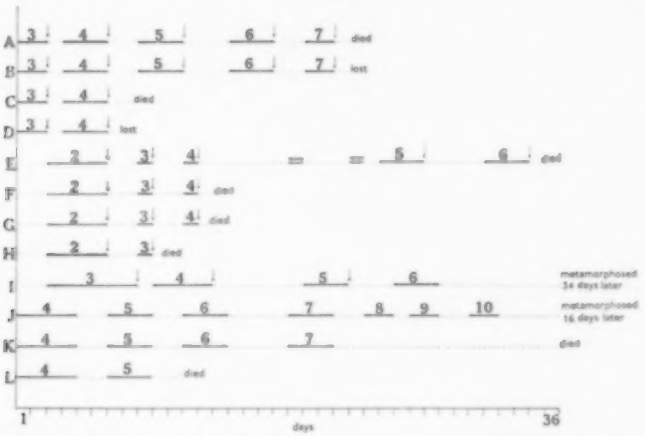


FIG. 2. Records of twelve larvae that were repeatedly dehydrated. All were received dry (8–12 per cent moisture content) from Northern Nigeria during 1959. —— Period drying in mud. ==== Period attached to walls of tube above the mud. ····· Period feeding in tap water. ↓ 2 hr in an electric oven held at 56–57°C.

In the third experiment four dry larvae were used. These were placed in water to feed for a few days. The second dehydration was done in mud in a glass tube. Only one (Fig. 2, I) survived the fourth dehydration. This larva was not exposed to more than 25°C at the end of the sixth dehydration. The larval–pupal ecdysis occurred 42 days after it was put to feed, and a female adult emerged 2 days later.

In the fourth experiment (Fig. 2, J–L) three dry larvae were placed in water to feed for a few days and were then dehydrated in mud in a glass tube. After each dehydration they were allowed to feed for a few days in water. At no time were they exposed to more than 25°C. One larva died after the fifth dehydration. One larva (Fig. 2, K) did not appear to be normal after the seventh dehydration, and it died 15 days after being placed in water. The third larva (Fig. 2, J) was dehydrated ten times. The larval–pupal ecdysis occurred 20 days after the tenth dehydration, and a female emerged on the twenty-first day.

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TOLERANCE TO REPEATED PARTIAL HYDRATIONS

Under natural conditions it may be supposed that the larvae are sometimes, especially in the dry season, subjected to slight or only very brief rains so that they are only partially wetted and then dried again before they have become active. The pharynx and heart began to beat regularly when the moisture content of the larva is increased to about 55 per cent, but the larva has to be much wetter than this before it can crawl or feed. The moisture content threshold for crawling and feeding has not been determined. When larvae with a moisture content of 8 per cent or less are placed in tap water, the pharynx and heart usually begin to beat in about 7–12 min at 25°C.

In preliminary experiments it was found that larvae would survive three partial hydrations that lasted only until the pharynx began to beat. Between each of these hydrations they were dehydrated to a moisture content of 8 per cent. In order to test the capacity of the larvae to survive more than three partial hydrations, three experiments were made using two, four, and ten larvae respectively. In all three experiments the larvae were dried on filter paper over calcium chloride or silica gel. Drying at room temperatures over these materials was found to reduce the moisture content of the larvae to about 4 per cent. Before being placed in water after the second and third dehydrations, they were heated in an electric oven to 56–57°C for 15 min. For the periods during which they were allowed to feed in water they were kept in Petri dishes in an incubator at 25 ± 1°C.

In the first experiment the heat treatment was applied at the end of the third and subsequent dehydrations. One larva failed to recover at the end of the eighth dehydration. The last six dehydrations of this larva were done within 10 hr, and it is therefore very probable that the larva was not sufficiently dry to escape damage when heated. The third to ninth dehydrations of the second larva took place within 11 hr. When placed in water at the end of the ninth dehydration, the pharynx did not beat even after 50 min although the dorsal vessel was beating regularly. This larva was then dehydrated over silica gel for 2 days before the next exposure to 56–57°C. When placed in water neither the pharynx nor the dorsal vessel beat, but after 50 min the larva responded feebly to touch for a few hours and then died.

In the second experiment (Fig. 3) using four larvae the heat treatment was applied at the end of the second and subsequent dehydrations. The second to tenth dehydrations were done within 5 days. After the tenth dehydration the pharynx and dorsal vessel of all four larvae beat regularly when they were placed in water. The larvae were left in tap water with food, but all died within 1 or 2 days.

In the third experiment (Fig. 4) using ten larvae the heat treatment was applied at the end of the third and subsequent dehydrations. In three larvae (Fig. 4, A–C) the pharynx beat when they were placed in water after the tenth dehydration. These larvae were left in water but died within 24 hr. After the ninth dehydration two larvae (Fig. 4, D–E) had opaque spots in the head although the pharyngeal beat was established in water. Local heat injury can usually be

easily recognized if severe because when the larvae are placed in water the affected area is opaque instead of more or less transparent. These two larvae were left in water, and both died in about 24 hr. Another larva (Fig. 4, F) was affected in the

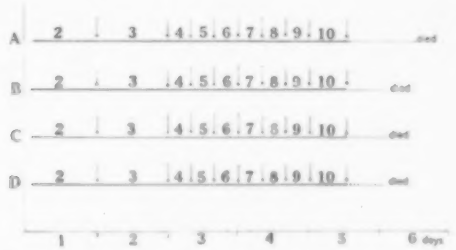

FIG. 3. Survival of four larvae immersed in tap water after the second and subsequent dehydrations only long enough for the pharynx to beat regularly. Before the experiment the larvae had been dehydrated to a moisture content of about 8 per cent after which they had fed for several days in water. Arrows indicate exposure to 56–57°C for 15 min in an electric oven. Partial hydrations were all done within a few minutes of removing the larvae from the oven. The moisture content of the larvae had been reduced in each instance to about 4 per cent before they were heated. Dotted lines indicate longer periods in tap water.

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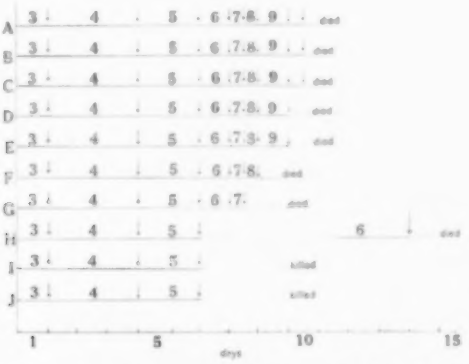

FIG. 4. Survival of ten larvae immersed in tap water after the third and subsequent dehydrations only long enough for the pharynx to beat regularly. Before the experiment all larvae had been dehydrated twice to a moisture content of about 8 per cent; after each of these two dehydrations they had been allowed to feed in tap water for 2 or more days. Arrows indicate exposure to 56–57°C for 15 min in an electric oven, but specimen 'H' was heated for 2 hr at 57°C after the sixth dehydration. Partial hydrations were all done within a few minutes of removing the larvae from the oven. The moisture content of the larvae had been reduced in each instance to about 4 per cent before they were heated. Dotted lines indicate longer periods in tap water.

same way after the eighth dehydration, and another (Fig. 4, G) after the seventh dehydration, but the latter survived for nearly 2 days during the early part of which time it was active and apparently took some food. One larva (Fig. 4, H) was allowed to feed in water for 5 days after the fifth dehydration, and it was then

dried over a period of 4 days in mud in a glass tube. At the end of this sixth dehydration it was heated for 2 hr at 57°C. It died about 24 hr after being placed in water. Two larvae (Fig. 4, I–J) left in water after the fifth dehydration seemed to be normal in every respect but were accidentally crushed and killed during an examination 3 days later.

TOLERANCE TO HIGH TEMPERATURES

(a) *Larvae in water*

It has been shown (HINTON, 1951, 1952) that active larvae tolerate water temperatures up to 41°C for continuous periods longer than any they encounter under natural conditions. They do not survive exposure to temperatures of over 43°C for more than about 1 hr. A few Chironomid larvae are known to tolerate temperatures above 40°C for prolonged periods, e.g. *Chironomus tentans* F. has been found living in a spring at 44·8°C in Yellowstone National Park, U.S.A. (BRUES, 1932). Some Stratiomyid larvae living in hot springs appear to tolerate temperatures up to 47°C (BRUES, 1932). Of the well-substantiated temperature records for insect larvae, perhaps the highest concerns the larva of the Ephydrid, *Scatella thermarum* Collin, which has been found living in water at 47·7°C in hot springs in Iceland (TUXEN, 1944).

(b) *Dehydrated larvae*

When the shallow pools in which the larvae live dry, the larvae remain in their tunnels in the mud and dry out as the mud dries. In the dry season the shade temperatures of some of the localities in which the species is found rise to a little above 40°C. The surface temperatures of fine soils may be 20–30°C above the shade temperatures. During the hottest part of the day the surface of the dry mud may therefore be as high as 70°C, or perhaps a little higher owing to radiation from the rock sides of the pools. However, the larvae are probably rarely exposed to such temperatures because the surface of the mud is usually protected by some plant debris, and most larvae are 4–8 mm below the surface. There is a steep fall in temperature away from the surface: even assuming a surface temperature of 80°C, the temperature at 4 mm is 70°C and at 8 mm it is 60°C (BUXTON, 1924). When dried larvae were exposed to similar temperatures, it was found (HINTON, 1951) that 93 per cent survived exposure to 61°C for 14 hr and 26 per cent survived exposure to 68°C for 11 hr. It therefore appears that the capacity of the larvae to tolerate high temperatures in the dehydrated state is more than adequate to meet the exigencies of their natural habitat.

As is well known, organisms and tissues that can tolerate more or less total dehydration can also tolerate temperatures far higher than any they are likely to encounter in their natural habitats. Thus, to an organism that can tolerate dehydration, which may be of great selective value, accrues a capacity that may be of little or no selective value. In a previous paper (HINTON, 1951) it was shown that larvae of *Polypedilum* recover temporarily when exposed to temperatures above 100°C. For instance, recovery for short periods was obtained after exposure

to 106°C for 3 hr, to 126–130°C for 21 min, and to 199–201°C for 5 min. Two of the eight larvae exposed to 199–201°C for 5 min made a temporary recovery. One of these lived for 21 hr. During this time it would respond readily to touch by making avoiding movements. It is thus clear that at least some of the sensory-motor pathways in the central nervous system remained intact, an indication that no very gross damage was done to the ganglia, nerves, or sensory end organs by exposure to 200°C for 5 min.

The results of further experiments on the temporary recovery and survival of dry larvae exposed to temperatures of over 100°C are summarized in Table 2. The moisture content of the larvae was first reduced to about 3 per cent or less

TABLE 2—TEMPORARY RECOVERY AND SURVIVAL OF LARVAE EXPOSED TO TEMPERATURES ABOVE 100°C

Preheating for 10 min (°C)	Temperature (°C)	Time (min)	No. of larvae	% recovery	Greatest life (hr)	No. metamorphosed
55–70	102–104	1	25	100	—	2
55–70	103–104	1	5	100	—	1
55–70	104	5	24	100	31	0
57	103–105	5	12	100	41	0
57	104–105	5	6	100	6	0
65	106	5	8	100	5	0
57	102–106	10	6	100	10	0
57	106	60	80	99	24	0

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with phosphorus pentoxide or other dehydrating agents, either at normal pressures or at a pressure of 10 mm Hg. They were then placed in an electric oven for 10 min at the temperatures cited in the first column. After this they were immediately transferred on to a watch glass in an oven held at over 100°C. The temperature of the watch glass in the second oven had reached equilibrium with the air temperature, so that no heat was lost from the larvae by conduction. The temperature ranges given in the second column of Table 2 are due to opening the oven door. For instance, in the first experiment cited the oven was held at 104°C, but by the time the door had been closed again the temperature had fallen to 102°C; it had risen to 104°C by the end of a minute. After the heat treatment, the larvae were either exposed to a relative humidity of 100 per cent for periods of 45 min to 3 hr before being placed in tap water, or they were dropped directly into the water. The percentage that temporarily recovered was not affected by this difference in treatment. The larvae that lived after the heat treatment were kept in Petri dishes in an incubator at 25 ± 1°C.

Of the twenty-five larvae subjected to 102–104°C for 1 min (Table 2), all were dead by the end of the third day after being placed in water. One larva died after feeding apparently normally for 12 days, one that appeared to be normal in every way was lost on the seventieth day, and two metamorphosed. One of the latter produced an apparently normal male adult after 24 days and the other a

female after 47 days. The female partly emerged from the pupal cuticle but was unable successfully to shed it and died a day later.

Of the five larvae subjected to 103–104°C for 1 min, four were dead within a day of being placed in water. The remaining larva produced a male adult 114 days later. This male was unable completely to shed its pupal cuticle and died within about 24 hr of attempting to do so. It is possible that some effects of heat injury are much delayed and affect the capacity of the adult to emerge from the pupa, since two of the three adults obtained by exposing larvae for 1 min to 102–104°C were unable completely to shed the pupal cuticle.

Temporary recovery of large fragments of larvae after exposure of temperatures of over 100°C seems to be as good as that of whole larvae. The six fragments used recovered temporarily: in the anterior fragments a pharyngeal beat was established and in the posterior a dorsal vessel beat. The fragments were treated before exposure to high temperatures as were whole larvae.

1 min at 102–104°C

(1) Head, thorax, and first five abdominal segments
(2) Head, thorax, and first two abdominal segments
(1) Abdominal segments 3–10
(1) Abdominal segments 5–10

5 min at 104°C

(1) Head, thorax, and first abdominal segment

TOLERANCE TO LOW TEMPERATURES

The resistance of active, hydrated larvae to low temperatures has not been investigated, but, as previously noted (HINTON, 1960), larvae with a moisture content of about 8 per cent survive immersion in liquid air (−190°C) and liquid helium (−270°C). In all experiments the larvae were transferred from room temperatures to those of the liquid gases: they were not pre-cooled. Two lots of five larvae each were dropped into liquid air and kept in it for 2 and 77 hr respectively. All of these larvae appeared to grow quite normally when subsequently placed in water. Larvae in a glass tube in a metal container were immersed in liquid helium. Five were kept in the liquid helium for 3 min and six for 5 min. All appeared to grow quite normally when subsequently placed in water. Two of the larvae treated with liquid air and two of those treated with liquid helium were kept and in due course produced adults.

SURVIVAL OF DRY LARVAE IN ETHANOL AND GLYCEROL

(a) *Survival in ethanol*

Active larvae die in less than 1 min when immersed in absolute alcohol, but when their moisture content is reduced to 3 or 4 per cent before immersion most recover at least temporarily. The fate of seventy-eight larvae immersed in various

concentrations of ethanol for periods of 24 hr or more is recorded in Table 3. For these preliminary experiments larvae were used that had been dried at room temperatures to a moisture content of about 8 per cent. Before immersion in ethanol the moisture content was reduced to 3 or 4 per cent by drying at 25°C

TABLE 3—TEMPORARY RECOVERY AND SURVIVAL OF DRY LARVAE IMMERSED FOR VARIOUS PERIODS IN DIFFERENT CONCENTRATIONS OF ETHANOL (18–20°C)

% ethanol	Time (hr)	No. of larvae	% recovery	Greatest life (hr)	No. metamorphosed
100	24	5	100	—	1
100	24	3	100	576	0
100	24	8	100	27+	0
100	24	2	100	22+	0
100	24	2	100	10+	0
100	24	6	83	12−	0
100	24	7	29	5+	0
100	48	7	100	51+	0
100	48	11	54	41−	0
100	168	4	100	55+	0
100	168	6	100	31+	0
95	24	5	100	6+	0
90	24	12	0	0	0

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over silica gel. After the larvae were removed from the ethanol, they were dried over calcium chloride or silica gel for several hours to about a day before being immersed in tap water. They were kept in water in Petri dishes in an incubator held at 25 ± 1°C. Larvae were considered to have recovered from the treatment with ethanol if after immersion in water the pharyngeal or dorsal vessel beat, or both, was established. In a very few of those entered in Table 3 as having recovered neither the pharyngeal nor dorsal vessel beat was established, but the larvae responded readily to touch.

Of the seventy-eight larvae treated with ethanol, only one survived. It had been immersed in absolute ethanol for 24 hr, and it produced an apparently normal adult female 72 days after being placed in tap water. Another larva of the same lot of five was active for 16 days after it was in water, when it was fixed for histological examination. During this time it appeared not to be feeding, and its fat body was very much diminished by the time it was killed. As shown in Table 3, another larva lived in water for 24 days after an immersion of 24 hr in absolute ethanol. This larva also appeared to have taken no food, and its fat body was also much reduced by the time it died. In one of the ten larvae immersed for 7 days in absolute ethanol the dorsal vessel was beating regularly 55 hr after immersion in water.

As expected, the toxicity of ethanol to the larvae was greatly increased by hydration, and no larvae recovered even temporarily when treated with 90%

ethanol. It seems probable that the figures (Table 3) for temporary recovery and survival would have been better had the larvae been drier before treatment with ethanol. The resistance of dry larvae to ethanol does not depend upon the failure of ethanol to penetrate through the body wall: there appeared to be no significant difference between the recovery of fragments of larvae and whole larvae immersed in ethanol. For instance, in one experiment a larva was cut in half between the third and fourth abdominal segments and both fragments were immersed in absolute ethanol for 24 hr. After drying in the usual way, they were placed in tap water. Within a few minutes the pharyngeal beat of one fragment and the heart beat of the other were established. In another experiment, a fragment consisting only of the head, thorax, and half of the first abdominal segment was immersed in absolute ethanol for 24 hr. This fragment responded readily to touch from the seventeenth to the thirtieth minute after being placed in water. In a third experiment a fragment consisting of abdominal segments 3–10 was immersed in absolute ethanol for 24 hr, and the pulse rate of the heart 45 min after being placed in water was 120/min. It may also be noted here that when the larvae dry they normally remain with the prestomum and preoral cavity open.

(b) *Survival in glycerol*

The recovery and survival of forty-three larvae immersed in pure glycerol for periods of 1–7 days is recorded in Table 4. The larvae used in these experiments had been dried at room temperatures to a moisture content of about 8 per cent.

TABLE 4—TEMPORARY RECOVERY AND SURVIVAL OF DRY LARVAE IMMERSED IN PURE GLYCEROL FOR PERIODS OF 1–7 DAYS (18–20°C)

Time (hr)	No. of larvae	% recovery	Greatest life (hr)	No. metamorphosed
24	4	100	—	2
67	6	100	—	1
168	12	100	—	1
168	11	100	96+	0
168	10	20	10+	0

Before immersion in glycerol the moisture content was reduced to 4 per cent or slightly less by drying over silica gel at 25°C. In the experiment with eleven larvae (Table 4), however, the moisture content had not been reduced below 8 per cent before treatment with glycerol. After the required period in glycerol (18–20°C), the larvae were removed with the aid of a forceps and dropped into a large beaker of water in which they were thoroughly shaken for 1–2 min to remove the glycerol as rapidly as possible. The criteria for recovery were those used in the experiments with ethanol. The pharyngeal beat was usually established about

10 min or so after immersion in water. The larvae were kept in water in Petri dishes in an incubator held at 25 ± 1°C.

Of the four larvae in glycerol for 24 hr, two died before metamorphosing, one at the end of the first day and the other after 103 days in water. During this time the latter fed and in every way appeared to be unaffected by the glycerol treatment. Of the two that produced adults, one did so after 72 days and the other after 100 days. Of the six larvae in glycerol for 67 hr, only two were alive 4 days after they were placed in water. One of these survived for 74 days during which time it fed and appeared to be unaffected by the glycerol treatment, and the other produced an adult 80 days later. Only one of the twelve larvae kept in glycerol for 168 hr was alive 7 days after they were placed in water. This larva produced an apparently normal female adult 50 days after it was immersed in water.

DISCUSSION

The term *cryptobiosis* has recently been proposed by KEILIN (1959, p. 166) to replace the terms anabiosis and abiosis. It is defined by him as, 'the state of an organism when it shows no visible signs of life and when its metabolic activity becomes hardly measurable, or comes reversibly to a standstill'. Since there is a clear qualitative difference between a very low level of metabolism and a total absence of metabolism, it is perhaps best to restrict the term cryptobiosis to a state when the metabolic activity of the organism comes reversibly to a standstill, as it necessarily does in those plants and animals that, for instance, grow after immersion in liquid helium.

Many workers have been reluctant to consider even the possibility of a temporary discontinuity in metabolic processes and have therefore held the view that however slow metabolic processes may become they are nevertheless proceeding continuously in order to supply the energy for the upkeep of the complex structure of the organism. But, as KEILIN (1953) points out, this view, which can hardly be accepted when extreme and protracted dehydration occurs, must be completely abandoned when one considers the fact that many organisms survive the complete solidification that occurs even before the temperature of liquid helium is reached.

In cryptobiosis all metabolic processes have ceased and chemical reactions, if any, are entirely adventitious: the organism has in fact been reduced to a purely morphological state, the successful maintenance of which does not require interaction between cells or between the constituents of cells but only requires that certain spatial relations be preserved. It is therefore not surprising that in a state of cryptobiosis irreversible mechanical injuries are tolerated that are totally incompatible with the requirements of the organism in an active state. They are also tolerated by organisms dehydrated nearly to a cryptobiotic state. For instance, it has been shown (HINTON, 1953) that larvae of *Polypedilum* in which all but the first two abdominal segments had been removed when dehydrated made a temporary recovery when placed in water 2 years after the injury had been inflicted. In short, mechanical injuries great enough to cause death in a few

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minutes can be inflicted on the organism in a state of cryptobiosis and its death can be postponed indefinitely so long as it remains in cryptobiosis.

Only tissues or organisms in a state of cryptobiosis, as redefined above, can tolerate very low or very high temperatures. For instance, recovery after a sojourn in liquid helium would seem to be indisputable evidence that the organism or tissue had been in a state of cryptobiosis. Recovery after exposure when dry to temperatures of over 100°C also seems to be certain evidence of a capacity to enter a state of cryptobiosis; and such organisms as have been found capable of tolerating when dry temperatures of over 100°C have also been found to tolerate immersion in liquid helium, e.g. nematodes, rotifers, tardigrades, and *Polypedilum*. In order to demonstrate a state of cryptobiosis, the capacity to tolerate very low or very high temperatures is easier to investigate, and the results are less open to question, than attempts directly to determine the absence of metabolism, which at best can only provide evidence of a negative kind.

The capacity of organisms when dry to tolerate very high temperatures, for instance temperatures over the boiling point of water, may at first sight appear to be less certain evidence of cryptobiosis than tolerance to very low temperatures. This is because at −270°C no chemical change occurs. Even at −200°C, BECQUEREL (1951) has calculated that if reactions are at all possible in the solid state of matter, they would be about 8×10^{-6} slower than at 20°C. On the other hand, the dry organism certainly undergoes chemical changes at high temperatures, as may be deduced from the fact that exposure to such temperatures must be brief if the organism is to recover and briefer still if it is to survive. But the oxidations that occur at very high temperatures can hardly be supposed to be metabolic: they would occur if the organism were dead and not in a state of cryptobiosis.

The capacity to tolerate a high degree of dehydration is usually certain evidence that cryptobiosis can be induced. In most arthropods the tolerated fall in moisture content usually appears to be not more than about 10–20 per cent, and the eggs of *Locustana pardalina* Walk. are very exceptional in that they are able to survive a fall in moisture content of about 63 per cent, i.e. from a moisture content of about 85 per cent to one of about 40 per cent (MATTHEE, 1951). Dehydration of the tissues may be so slowed down by the impermeability of the embryonic membranes, chorion, or cuticle, or by the production of metabolic water, or both, that the animal may survive desiccation for many years, e.g. the larva of the South American coccid, *Margarodes vitium*, was found to be alive after at least 17 years in a museum (FERRIS, 1919), and the development of the larva of the North American wood-boring beetle, *Eburia quadrigeminata*, has been delayed up to 40 years in dry wood (JAQUES, 1918). But however resistant most arthropods may be to desiccation, they tolerate only a certain fall in their moisture content: sooner or later a critical level is reached below which they cannot survive. A few arthropods, however, differ qualitatively in this respect: at physiological temperatures there is no apparent limit to the dehydration they tolerate, e.g. tardigrades, eggs of some crustacea (Branchipoda, Copepoda, Ostracoda), and the

larva of *Polypedilum*, and in all of these cryptobiosis can be induced. From a consideration of the minimum moisture content that can be tolerated by animals that do not tolerate more or less total dehydration, it may be predicted with considerable confidence that if an animal will tolerate a moisture content of, say, 20 per cent it will almost certainly tolerate more or less total dehydration and can become cryptobiotic.

Although diverse groups of invertebrates, including a number of arthropods, are capable of tolerating complete dehydration, amongst insects it is only the larva of *Polypedilum vanderplanki* that is definitely known to survive such conditions. It seems probable that the larva of the Mycetophylid, *Sciara medullaris* Giard, can also survive complete dehydration, judging from the account given by GIARD (1902); and there is some circumstantial evidence that a Ceratopogonid larva that occurs in the same environment in Africa as *Polypedilum* may have to be included in the list. From the fact that a few unrelated groups of arthropods can survive complete dehydration, it seems evident that this capacity has arisen independently in each group, and perhaps independently on several occasions within the Insecta. But if the capacity of the whole insect to survive dehydration proves to be restricted to only a few species, as it now appears to be, the capacity of individual tissues in the insect to survive complete dehydration would seem by no means so restricted. Thus, many insects that at physiological temperatures cannot survive a fall in moisture content of more than about 20 per cent may nevertheless possess tissues that can tolerate complete dehydration, as has been claimed for several species of Coleoptera and Diptera by HINTON (1957). In these Coleoptera and Diptera the epidermis appears to survive complete dehydration. Its appearance before and after dehydration is identical. When placed in water after a month over phosphorus pentoxide tanned wound plugs were formed where the epidermis was pricked. Even after heating when dry to 103°C for 1–2 hr, a similar reaction could be obtained in the re-hydrated tissue. Evidence of this kind that the tissue is alive is open to objection. For instance, it may be claimed that the epidermis was not alive and that damage to it simply destroyed a barrier between enzyme and substrate that enabled the tyrosinase reaction to proceed. However, SELMAN (1961) has produced unexceptionable evidence that in the larva of *Sialis lutaria* L. (Megaloptera), an insect that does not tolerate complete dehydration, certain blood cells nevertheless survive the treatment: the coagulocytes clot and form pseudopodia after dehydration as they do before dehydration. The typical clotting behaviour of the blood cells occurs even after dehydration for 17 months and heating when dry to 100°C for 30 min.

The results of the experiments cited in the preceding paragraph seem to show that cryptobiosis can be induced in certain individual tissues of many species of insects by exposure to conditions that rapidly kill the insect as an organized whole. Such results perhaps also suggest that with the discovery of suitable techniques the metabolism of many insects and not just that of some of their tissues may be brought reversibly to a standstill. In this connexion the experiments of ASAHINA and AOKI (1958) are of particular interest. By careful pre-cooling,

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these writers have been able to secure apparently normal growth in larvae of *Cnidocampa flavescens* Walk. (Eucleidae) and *Aporia crataegi* L. (Pieridae) after immersion for 24 hr in liquid oxygen.

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THE NUTRITIONAL REQUIREMENTS OF LOCUSTS—III CARBOHYDRATE REQUIREMENTS AND UTILIZATION

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Abstract—When carbohydrate other than indigestible cellulose was omitted from an otherwise satisfactory diet the locusts *Schistocerca gregaria* (Forsk.) and *Locusta migratoria* L. grew poorly and failed to complete development. Normal growth was obtained when glucose or sucrose formed 13 per cent of the diet, but some experiments indicated that this percentage was inadequate for good development beyond the third instar. Satisfactory growth and development occurred with 26 per cent carbohydrate. Thirty-nine per cent glucose or sucrose allowed complete development but retarded growth throughout, and with 6 per cent or less, growth was severely curtailed from the start and few individuals survived beyond the third instar. It is shown that these growth differences could be only marginally a result of differences in palatability caused by sugars, and it is therefore concluded that carbohydrate is nutritionally essential for locusts. There is some indication that the requirement for it increases during larval development. The utilizability of a representative series of carbohydrates and of some related substances was tested. Four pentoses were non-utilizable. Except for sorbose and galactose, all hexoses, oligosaccharides, dextrins, and starches to be tested were utilized, as were three sugar alcohols. Oil, fatty acids, or glycerol could not replace carbohydrates, and the substitution of extra protein had a deleterious effect.

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INTRODUCTION

ALTHOUGH some dipterous larvae have been reared on diets lacking carbohydrates (KADNER and LAFLEUR, 1951; SEDEE, 1953) most insects need it in their food (TRAGER, 1953; LIPKE and FRAENKEL, 1956; FRIEND, 1958). In synthetic diets this requirement has usually been met by the inclusion of glucose, sucrose, starch, or dextrin. Generally speaking little attention has centred on the relative value of other carbohydrates in the growth of larvae, although extensive utilization studies were early made on certain adult Hymenoptera and Diptera in which the only food required for normal longevity is sugar (PHILLIPS, 1927; VOGEL, 1931; FRAENKEL, 1940; HASSETT, 1948; HASSETT *et al.*, 1950; GALUN, 1955). In such cases metabolic requirements other than a suitable energy source are provided for during larval life, and utilization may be evaluated very simply in terms of longevity.

These studies showed that on the whole the same simple sugars were utilized by honeybees and various adult flies, and that the availability of complex carbohydrates further depended on the presence of suitable enzymes in the digestive fluid. The ability of various sugars to prolong survival of larvae of *Apis* and

Calliphora was assessed by similar methods (BERTHOLF, 1927; FRAENKEL, 1940), but for growth, requirements might be different.

Using synthetic diets normal growth and development of the flour-dwelling beetles *Tribolium*, *Stegobium*, and *Oryzaephilus* was obtained on a wide range of pentoses, hexoses, di-, tri-, and polysaccharides, only minor differences between the three species being found (BERNARD and LEMONDE, 1949; LEMONDE and BERNARD, 1953). In contrast the lepidopterous rice-borer, *Chilo suppressalis* grew well only with sucrose, maltose, fructose, and glucose, partly because it failed to utilize most monosaccharides, and additionally because of a restricted complement of digestive carbohydrases (HIRANO and ISHII, 1957). Comparison of the composition of food plant and faeces for several lepidoptera and the grasshopper *Melanoplus* have shown a frequent inability to digest starch and similar polysaccharides in phytophagous insects (BROWN, 1937a, b; CROWELL, 1941; EVANS, 1939a, b).

This paper deals with the carbohydrate requirements of two species of locust. Using a synthetic diet the optimum proportion of glucose or sucrose was determined, and the effect on growth of various substitutes is described.

TABLE 1—COMPOSITION OF THE BASIC SYNTHETIC DIET

Cholesterol	50 mg
Linoleic acid (B.D.H. technical grade)	0·2 ml
Casein, fat and vitamin free (B.D.H.)	6·0 g
Bacteriological peptone (B.H.D.)	2·0 g
Egg albumen powder (B.D.H.)	2·0 g
Salt mixture* (Glaxo DL.6)	1·5 g
Cellulose powder (Light & Co. chromatographic)	15·0 g
Carbohydrate†	10·0 g
Ascorbic acid	100 mg
Vitamins in 10 ml of 20% ethanol:	
Thiamine	25 μg/g diet
Riboflavin	25 μg/g diet
Nicotinic acid	100 μg/g diet
Pyridoxine	25 μg/g diet
Folic acid	25 μg/g diet
Inositol	250 μg/g diet
Pantothenic acid	50 μg/g diet
p-Aminobenzoic acid	25 μg/g diet
Choline chloride	1250 μg/g diet
Biotin	1 μg/g diet

* For composition see previous paper in this series (DADD, 1960a).

† White and yellow dextrin were supplied by Hopkins & Williams. Potato and soluble starch by B.D.H. All sugars apart from glucose and sucrose (B.D.H.) were supplied by L. Light & Co.

MATERIAL AND METHODS

Egg pods of *Schistocerca gregaria* (Forsk.) and *Locusta migratoria* L. were supplied ready to hatch by the Anti-Locust Research Centre, London. On hatching, hoppers were used in growth experiments set up as described elsewhere (DADD, 1960a).

The composition of the basic diet used in these experiments is set out in Table 1. Where different levels of carbohydrate were used, reciprocal adjustments were made to the amount of cellulose powder. Though itself a carbohydrate, cellulose is not digested by locusts, and functions in the diet as an inert, bulk material; it is disregarded in subsequent discussion. By thus balancing changes in carbohydrate concentration, the concentrations of all other nutritionally active components of the diet were at a constant level throughout.

The results of some ancillary tests on the phagostimulant properties of sugars are given in this paper where they bear on the interpretation of growth trials. The methods used are briefly noted at the appropriate juncture in the text.

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RESULTS

In previous work the carbohydrate of the diet given in Table 1 had usually been provided as equal parts of sucrose and dextrin, although it was known that sucrose alone could be used. Moreover, half this percentage of sucrose (i.e. 13 per cent instead of 26 per cent) when used in diets of a different protein constitution had allowed good growth and development of *Schistocerca*. It was therefore assumed that although 13 per cent carbohydrate might not be optimal, it would allow sufficiently good growth to distinguish substances which were utilized from those which were not. A major consideration in choosing the percentage of carbohydrate for diets used in utilization tests was the need to economize on the consumption of several expensive sugars. Moreover, there were grounds for suspecting that the high carbohydrate level, if given entirely as sugar, might adversely affect the palatability of diets. Consequently the lower level of 13 per cent was initially adopted; it subsequently became evident that this was inadequate for growth during the late instars, and the final experiment used diets with double this concentration.

Four experiments were made with hatchlings of each species, and the results are illustrated graphically using mean growth curves for each test diet. The first experiment comprised two series of diets with sugar (glucose or sucrose) at various concentrations, together with others containing various sugars at a concentration of 13 per cent. Growth curves for the glucose and sucrose concentration series are shown in Fig. 1, (a) and (b), and all diets with 13 per cent carbohydrate are compared in Fig. 2. As none of these latter supported normal growth during the final instar, though up to this stage performances were good with several of them, only growth curves for the early part of the larval period are shown. In all cases where the diet contained 26 per cent carbohydrate growth was normal throughout.

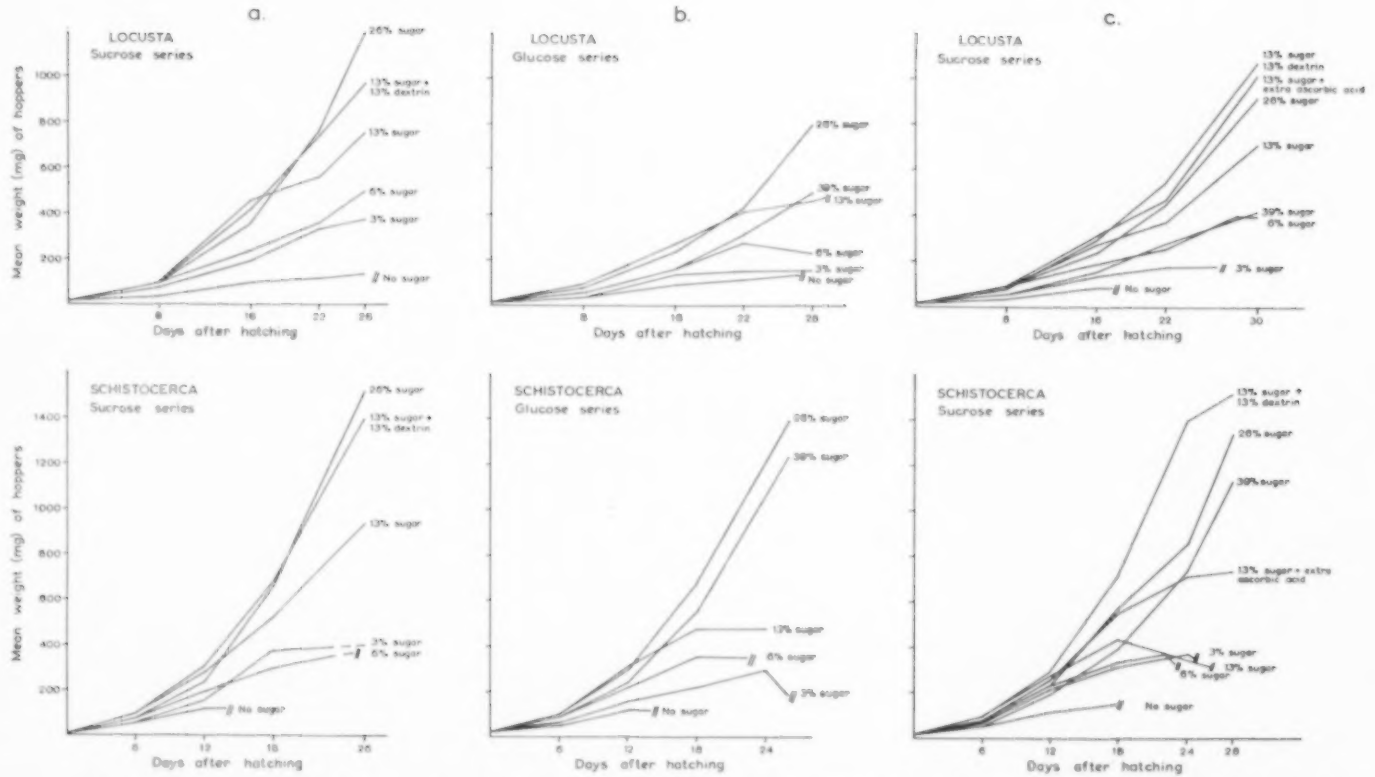

FIG. 1. The effect of various concentrations of carbohydrate (sucrose and glucose) on the growth of *Locusta* and *Schistocerca*. In this and subsequent figures a broken line indicates that less than 10 per cent of the hoppers alive on the sixth day after hatching were surviving; vertical lines at the end of a curve indicate that all hoppers had died.

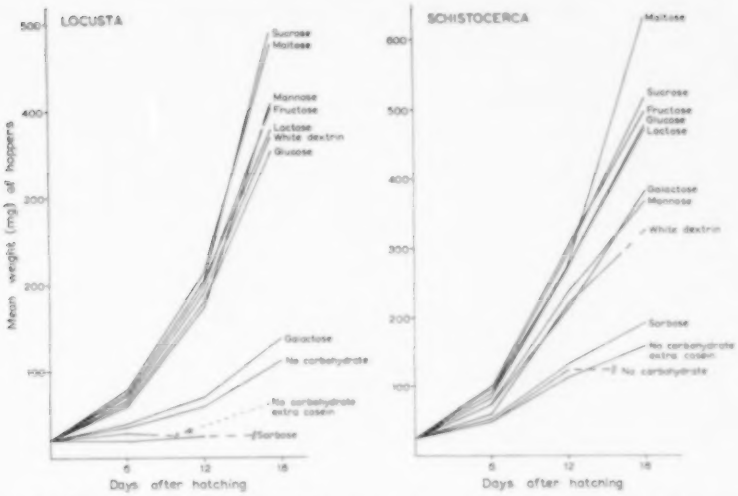

FIG. 2. Growth of *Locusta* and *Schistocerca* with various carbohydrates at a level of 13 per cent of the diet.

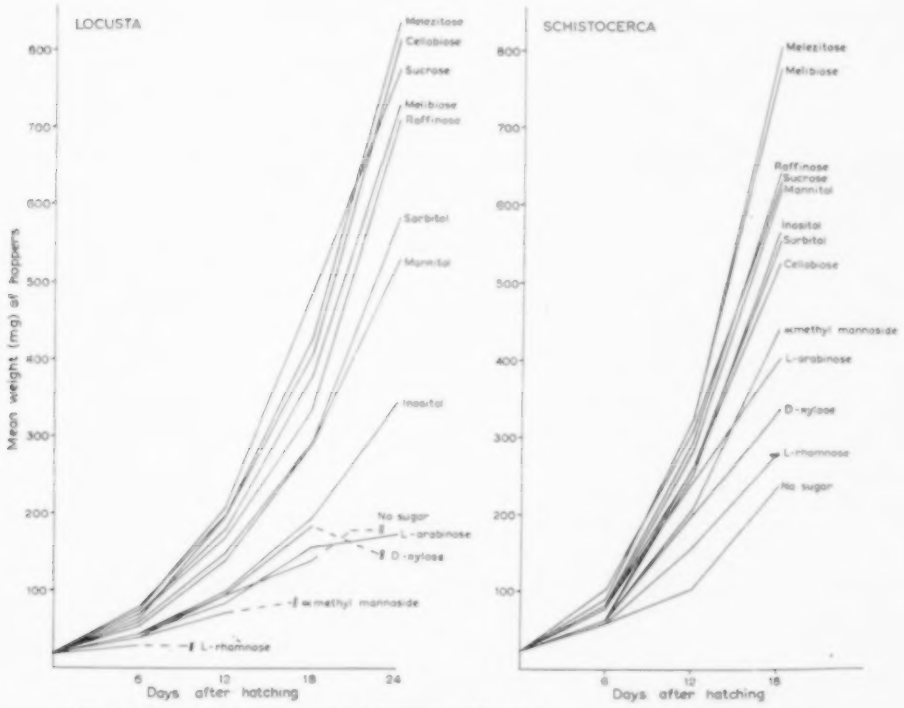

FIG. 3. Growth of *Locusta* and *Schistocerca* with various sugars and related substances at a level of 11 per cent of the diet.

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In the second experiment a variety of sugars and related substances were tested at the reduced level of 11 per cent of the diet, as only small quantities of some of them were available. Mean growth curves for this group of diets are shown in Fig. 3. In general, performances were much better than had been the case in the previous experiment, but again growth tended to fall off during the fifth instar. As complete development was obtained on some of the diets containing only 11 per cent sugar, the various concentrations of sucrose previously used were again tested to check the relative superiority of the 26 per cent compared with the 13 per cent diet in the late instars. The results, shown graphically in Fig. 1 (c), fully confirmed the earlier experiments for both species; good growth throughout occurred on the diets with 26 per cent carbohydrate, but although initially 13 per cent seemed adequate, growth during the fourth and fifth instars became retarded. A few small *Locusta* adults were obtained, but no *Schistocerca* hoppers survived the fifth instar.

It thus appeared that either the requirement for carbohydrate changes in the course of larval development, a situation which occurs with the larvae of *Pyrausta* (BECK, 1956); or that in the presence of low amounts of sugar some other essential nutrient in the diet decayed and gave rise to poor growth towards the end of the experiment. The latter possibility came to mind for two reasons. Owing to the large numbers of diets to be compared, enough food to last the entire experiment was prepared and stored at the start; hitherto diets had been prepared afresh every 10 days or so. Secondly, the failure of growth at the fifth instar resembled the situation which occurred on diets deficient in ascorbic acid (DADD, 1960b), and it was found on testing several old diets that, in fact, little trace of ascorbic acid could be detected after a month's use. In repeating the experiment with the sucrose concentration series diets were prepared afresh after 10 days to lessen the possibility of deterioration, and a 13 per cent sucrose diet with additional ascorbic acid was tested. This gave improved growth with *Locusta* and some of the hoppers in this group became adult; with *Schistocerca* growth appeared slightly improved but no adults were obtained (Fig. 1, c). The results for *Schistocerca* were in accord with the hypothesis that an increased requirement for carbohydrate occurred in the late instars even though some improvement followed augmentation with ascorbic acid. The results for *Locusta* suggests that the improved growth obtained with the higher (26 per cent) sucrose diet might have resulted from a sparing action on ascorbic acid.

These possibilities were further examined in an experiment in which *Schistocerca* hoppers were taken from stock culture (where they had been fed on grass) at the third instar and transferred to diets containing the various concentrations of sucrose used previously. If the higher percentage of carbohydrate were needed at this stage, growth on the 26 per cent diet might be expected to surpass that on the lower concentrations from the start of the experiment. It will be seen from the mean growth curves given in Fig. 4 that although growth was at all times slightly ahead on the 26 per cent diet, in fact little real difference occurred between the diets with 5–39 per cent of sugar until the fifth instar was reached. It was only at the

termination of the final instar that the inadequacy of diets with 13 per cent or less sugar became fully apparent; whereas most of the hoppers given 26 per cent and 39 per cent sucrose moulted perfectly and continued growth as adults, those given less moulted imperfectly or died shortly after fledging. In this experiment failure at the final moult was unlikely to have been due to decay of ascorbic acid, for it was known that third instar hoppers of *Schistocerca* reared on grass could thereafter

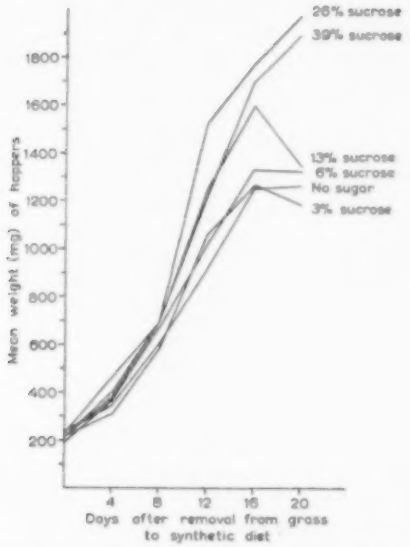

FIG. 4. The effect of different concentrations of sucrose on the growth of *Schistocerca* hoppers taken from grass diet at the third instar.

be reared to adults on artificial diets lacking ascorbic acid, presumably because adequate amounts are accumulated during early growth (DADD, 1960a). If an increased requirement for carbohydrate was in question, these results indicate that an accumulation of carbohydrate reserves in the early instars might likewise delay the expression of a later inadequacy.

This experiment incidentally showed that sugar, or other carbohydrate, was not needed in the food as a phagostimulant to ensure its ingestion in adequate amounts, for initially growth on the sugar-free diet was very little retarded, and was effectively the same throughout as on the diet containing 3 per cent sugar. It is appropriate at this juncture to describe experiments aimed at assessing the phagostimulant function of sugars and to consider their bearing on the results of growth trials.

In these experiments *Schistocerca* fifth-instar hoppers, deprived of food overnight, were caged in groups of four, with a water supply and weighed quantities of either pure cellulose powder or mixture of cellulose and sugar in various proportions. After 2 hr individual weight increases were recorded, and after 24 hr

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the faecal pellets produced and the amount of food taken were weighed. Using glucose/cellulose mixtures the data of Table 2 were obtained.

It is apparent that although appreciable quantities of pure cellulose were eaten, sugar caused increased ingestion (Table 2, row 3). As no faecal pellets were passed during the first 2 hr of the experiment, increments in body weight over this period represent the combined weights of solids and water to be ingested.

TABLE 2—AMOUNTS OF MIXTURES OF GLUCOSE AND CELLULOSE POWDER EATEN BY FIFTH-INSTAR HOPPERS OF *Schistocerca gregaria*. (For further explanation see text.)

	Percentage of glucose in mixture					
	None	2	5	10	20	40
Increase in weight (mg) per hopper after 2 hr	46	58	50	120	80	170
Weight (mg) of faecal pellets per hopper after 24 hr	34	62	88	88	53	22
Weight (mg) of mixture eaten per hopper in 24 hr	150	323	421	442	336	158

If the magnitude of these are taken as some indication of the strength of the immediate feeding response, it seems that there is a tendency for this to increase with the concentration of sugar (Table 2, row 1). On the other hand maximum ingestion of solids, measured directly by loss from food after 24 hr (Table 2, row 3), occurred with sugar concentrations of 5 and 10 per cent; with 2 and 20 per cent sugar, about three-quarters the amount was taken, while with 40 per cent ingestion was no more than for pure cellulose. Taking faecal production as an alternative index of solids consumed, a comparable pattern of ingestion is shown (Table 2, row 2), but it must be borne in mind that the use of faecal weights for this purpose takes no account of the variable utilizability of the different mixtures nor of the possibility that they might be retained in the gut for variable periods. For example, with 40 per cent sugar, the very high immediate increase in body weight, the low value for solids actually taken during 24 hr, and the low amount of faeces produced suggest that, with high utilizability, after an initial burst of feeding little egestion of material from the gut took place and further feeding was restricted. If this interpretation is correct, the low consumption observed with 0 and 40 per cent sugar may be the result of different limiting factors. In the first case a lack of phagostimulation is envisaged; with 40 per cent sugar phagostimulation was clearly strong, at least initially, and the failure to continue eating might perhaps be due to an inability of the *gut* to deal with a mixture containing excessive sugar.

However, for the present purpose this experiment indicated that sugar exerted its maximum effect on ingestion when it formed 5–10 per cent of a powdery mixture and was almost as effective over the range 2–20 per cent. It was therefore unlikely

that poor growth on diets differing only by the concentrations of the sugar within this range would be due to inadequate feeding. This being so the poor performance obtained in growth trials with diets low in sugar could be considered a valid expression of strictly nutritional deficiency.

In the final experiment a number of sugars previously used were retested, together with several additional substances, at the 26 per cent level. As before, growth curves for the early stages only are shown in Fig. 5, for in spite of the

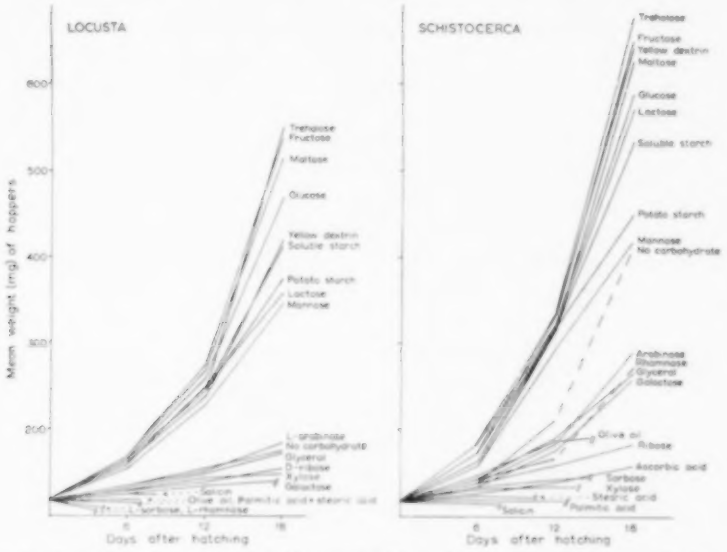

FIG. 5. Growth of *Locusta* and *Schistocerca* with various carbohydrates or substitutes at a level of 26 per cent of the diet.

high concentration of carbohydrate, growth was in all cases poor in the fifth instar. This was so for the diet containing glucose, which in the first experiment has been adequate throughout, and it must be supposed that some factor apart from the carbohydrate content was at fault. In this experiment, again owing to the large number of diets compared, all food was prepared before setting up the trials and eked out to the end. However, the aim of these comparisons was primarily to distinguish substances which were utilized from those which were not, and this was in most cases clearly apparent during the early stages of growth. With the proviso that where good utilization occurred in the early instars these experiments do not in most cases allow conclusions to be drawn about later growth, the following points may be made.

In the first experiment (Fig. 2) *Locusta* grew normally during the early instars with sucrose, maltose, D-mannose, fructose, white dextrin, and glucose. Galactose and L-sorbose afforded no better growth than a diet devoid of carbohydrate, the latter (sorbose) being if anything, inhibiting. Normal growth of *Schistocerca*

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occurred with maltose, sucrose, fructose, glucose, and lactose. Galactose, D-mannose, and white dextrin gave somewhat poorer growth, but were undoubtedly utilized. L-Sorbose gave no better growth than the carbohydrate-free control. With both species, a diet in which carbohydrate was replaced by additional casein (instead of additional cellulose) gave poorer growth than the normal control.

The second experiment (Fig. 3) showed that *Locusta* grew normally with melezitose, cellobiose, sucrose, melibiose, and raffinose, whereas growth was slightly retarded with sorbitol and mannitol; adults were obtained in all these cases. L-Arabinose, D-xylose, L-rhamnose, and α-methyl-mannoside afforded no better growth than a diet lacking carbohydrate. Meso-inositol at first seemed ineffective, but whereas hoppers on diets lacking utilizable sugars mostly died without reaching the fourth instar, those given inositol continued growth through the fifth instar and several made abortive attempts to moult to adults. Some utilization must therefore have occurred. Corresponding results for *Schistocerca* (Fig. 3) show that normal growth occurred initially with melezitose, melibiose, raffinose, sucrose, mannitol, inositol, sorbitol, and cellobiose; of these all except cellobiose, melezitose, and sorbitol produced adults. With α-methyl-mannoside growth was initially slow, but from the third instar it improved and adults were eventually obtained. This substance was therefore utilized, though slowly, by *Schistocerca*. Mean growth curves for the pentoses are difficult to interpret, for although they were better than the control and survival was markedly greater, individual performances overlapped. When retested at double the concentration (in the following experiment) none of these sugars afforded better growth than the carbohydrate-free control although survival was enhanced.

Several non-carbohydrates were included in a final experiment where test substances were incorporated as 26 per cent of the diet. The effectiveness of lipids in replacing carbohydrate was examined by using two fatty acids and olive oil. Glycerol and ascorbic acid, which have affinities with sugars, were tested, as were two available glycosides, rutin and salicin. In this experiment (Fig. 5) *Locusta* grew normally to the fifth instar with maltose, trehalose, fructose, glucose, yellow dextrin, and soluble starch; D-mannose, lactose, and potato starch gave as good growth initially, but failure occurred in the fourth instar; however, they were undoubtedly utilized. With L-arabinose, D-xylose, D-ribose, galactose, and glycerol little growth occurred and performances were essentially the same as on the control. As before L-sorbose seemed inhibitory, and at this high concentration L-rhamnose appeared so as well; with these two sugars mortality was complete before the sixth day. All the other substances tested apparently inhibited growth; mortality was complete by the twelfth day with olive oil, palmitic, stearic and ascorbic acids, rutin, and salicin. Early growth of *Schistocerca* (Fig. 5) was normal with trehalose, fructose, yellow dextrin, maltose, glucose, lactose, soluble starch, potato starch, and D-mannose, though the last two substances failed rather earlier than the others. Growth with L-arabinose, L-rhamnose, galactose, and glycerol was effectively the same as for the carbohydrate-free control, although survival

was markedly better with the three sugars. It is interesting to note that at this high concentration galactose appeared ineffective in growth, whereas previously it was definitely, although not optimally, utilized. D-Ribose afforded very little growth though survival was good. L-Sorbose again appeared to be inhibitory, as did D-xylose at this high concentration. All other substances tested, were, if anything, inhibitory.

DISCUSSION

It has frequently been observed in work with adult bees and flies that nutritionally adequate sugars might appear ineffective in survival tests owing to their failure to stimulate inhibition, while non-nutritive sugars have sometimes been found to have excellent phagostimulant properties (VOGEL, 1931; HASSETT, 1948; HASSET *et al.*, 1950). In larval growth studies on complete synthetic diets this twofold function of dietary sugar might be a troublesome source of misinterpretation, for where the natural gustatory stimuli are generally unknown, the possibility of sugar subserving the function of an artificial stimulant for inducing feeding is most likely to be encountered. Supposing this to be the case, poor growth on omission or reduction in the amount of sugar might be as much due to inadequate feeding as to nutritional inadequacy. Only measurement of food intake could distinguish between these possibilities, and this has generally been neglected in protracted larval growth tests, although its importance has been recognized in studies on adult flies (LIPKE and FRAENKEL, 1956). Failing this information the significance of attempts to grade sugars in terms of relative utilizability is doubtful.

Sugars are widely acceptable to insects, and with larvae of *Schistocerca* sucrose and glucose were found to be amongst the components of synthetic diets to have the most markedly phagostimulant properties when tested alone (DADD, unpublished data). It therefore seemed possible that lack of sugar might adversely affect growth through loss of palatability. However, as the absence of carbohydrate from an otherwise complete diet caused little initial retardation in the growth of third-instar hoppers of *Schistocerca*, it must be supposed that in this case hunger is a phagostimulant that may override other factors. The amounts of various mixtures of sugar and cellulose powder eaten by *Schistocerca* larvae indicated that maximal ingestion was induced by concentrations of sugar which in otherwise adequate diets allowed only poor growth. It was justifiable therefore to regard such growth failure as largely the expression of a strictly nutritional requirement for carbohydrate, although with the extreme concentrations used impaired feeding may have contributed to the results obtained.

The use of different concentrations of dietary sugar revealed an interesting situation in relation to the late larval growth of both species. Whereas 13 per cent of either sucrose or glucose allowed satisfactory growth in the early stages, additional carbohydrate, either sugar or dextrin, was required for satisfactory growth and development after the fourth instar. The considerable retardation in growth throughout caused by 39 per cent sugar may have been the result of poor feeding, for it seemed that at concentrations of more than 20 per cent sugar,

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ingestion of sugar/cellulose mixtures was sub-optimal. The differences in late larval growth could be taken to indicate either an increased requirement for carbohydrate at this stage, or that additional carbohydrate exerted a sparing action on some other nutrient which was otherwise in short supply. The weight of evidence from *Schistocerca* favoured the former hypothesis, but the results obtained by augmenting a 13 per cent sucrose diet with extra ascorbic acid, particularly those for *Locusta*, suggested that secondary interactions with other components of the diet might well be involved. Radical changes in carbohydrate requirements during development are well known in many holometabolous insects, and have been recorded during the growing stages of the European corn-borer *Pyrausta*, which develops normally during the first three instars without carbohydrate (though it preferentially feeds where sugar is present), but requires it thereafter (BECK, 1956). The changed demand is thought to be connected with the accumulation of reserve fat-body for the future adult; it would be of interest to know whether an analogous accumulation of reserve material occurs in the late instar of locusts.

In their ability to use various carbohydrates for growth *Schistocerca* and *Locusta* differ only in minor points. None of the pentoses tested were utilized by *Locusta* and were of little if any value to *Schistocerca*. Both species grew well on all the hexoses with the exception of sorbose and galactose; *Schistocerca*, however, seemed able to utilize the latter to some extent at the lower concentration tested. Starches, dextrins, and all oligosaccharides supported good growth in both species. From this it is clear that the digestive fluid of locusts must contain an amylase complex and an extensive set of glycosidases. It was in fact possible to hydrolyse all the oligosaccharides used in these experiments *in vitro* with an extract of the midgut of adult *Locusta* (MURRAY, private communication).

The sugar alcohols mannitol and sorbitol allowed good growth of both species, glycerol was of no value to either and the cyclic hexanol meso-inositol supported normal growth in *Schistocerca* and was utilized, although with difficulty, by *Locusta*. The role of inositol in replacing carbohydrate is of interest as it is also required by locusts (DADD, 1961) and a few other species (FORGASH, 1958; VANDERZANT, 1959), in micro-quantities, as a growth factor.

An apparent difference between the two species related to α-methyl-mannoside; this enabled *Schistocerca* to grow to the adult stage, although slowly, whereas it appeared to inhibit growth in *Locusta*. Whether this inhibition was caused by toxicity or a gustatory repellence sufficiently powerful to induce starvation cannot be determined in the absence of feeding data. Similarly the early mortality of both species on diets containing stearic, palmitic, and ascorbic acids, and the glycosides rutin and salicin, can be interpreted equally well in terms of toxicity or a failure to eat.

Apart from these substances, certain sugars, notably sorbose, appeared inhibitory rather than merely inert. Of interest in this connexion is the inhibition observed in the growth of *Tenebrio* with all monosaccharides which were not utilized (FRAENKEL, 1955). This was taken to indicate that the sugars in question

were toxic, but in the absence of information on the amounts of food eaten it is not readily apparent that palatability may not have been involved.

Olive oil was unable to replace carbohydrate for either species. It is unlikely that palatability was adversely affected, for it has been shown that for adult *Schistocerca* olive oil is a phagostimulant (CHAUVIN, 1951). The ineffectiveness of oil is of interest in view of the apparent ability of *Schistocerca* to utilize body fats directly as an energy source in flight (WEIS-FOGH, 1952).

The utilization of carbohydrates in the growth of other insects for which information is available has been tabulated by HIRANO and ISHII (1957). Generally speaking, pentoses are of doubtful value as they are for locusts. In dealing with hexoses, locusts are apparently more versatile than the other species studied. Apart from locusts, mannose was available only to *Tribolium* and *Oryzaephilus*; it was considered toxic with *Tenebrio*, a property it also has with the adult bee (VOGEL, 1931). Galactose is generally not well utilized. *Schistocerca* grew fairly well with it in low concentration, but grew no better than on a sugar-free diet when this concentration was doubled. Anomalous results have been obtained with galactose in survival studies of adult *Drosophila*, where some individuals could utilize it and others not (HASSETT, 1948). Sorbose is generally not utilized, the positive result recorded for *Stegobium*, being doubtful (FRAENKEL, 1955). Sorbitol and mannitol were utilized in all cases studied. No comparative information is available on the value of inositol as a carbohydrate in larval growth, but it was utilized to some extent in prolonging the longevity of adult *Drosophila* (HASSETT, 1948).

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In their ability to metabolize a wide array of di-, tri-, and polysaccharides locusts most nearly resemble *Tenebrio*, and like this insect must be furnished with an extensive complement of carbohydrases, including the unusual β-glycosidases necessary to account for the digestion of cellobiose and lactose. They stand in marked contrast to *Chilo*, the only other phytophagous insect studied, which grew optimally only on glucose or fructose.

A somewhat surprising result was the good growth obtained with potato starch, for in the acridid *Melanoplus* comparative analyses of food and faeces showed little digestion of polysaccharide (BROWN, 1937a, b). It is possible perhaps, that in the preparation of starch, processing might render the native starch granules amenable to enzyme attack.

It is unfortunate that time precluded repetition of the tests with sugars at the high concentration. As they stand, little can be said of growth in the late instars, for failure of the glucose control showed that some unknown deficiency, probably connected with overlong storage, had intervened. But it might have proved the case that of those sugars which allowed normal initial growth, only some might have sufficed throughout, a situation which could be envisaged were an increased demand in the late larval period to arise from different metabolic processes. Moreover, as it has been shown that in *Schistocerca* the rate of uptake of certain hexoses (glucose, fructose, and mannose) and their conversion to trehalose varies considerably (TREHERNE, 1958), it might well be the case that even were no

fundamentally different metabolic pathway involved in the increased demand for carbohydrate, certain sugars would be of no avail through their failure to be absorbed at an adequate rate.

An interesting problem awaits investigation here. But in spite of uncertainties about their complete adequacy, it is clear that a very wide range of carbohydrates and related substances may be utilized by locusts, which in this respect are the peers of those carbohydrate specialists, the stored-products insects.

SUMMARY

1. The effect on the growth of *Schistocerca* and *Locusta* of different percentages of sugar (sucrose or glucose) in a synthetic diet otherwise devoid of digestible carbohydrate was examined. Similar results were obtained with both species. With 5 per cent or less sugar, growth was poor from the start and few individuals survived beyond the third instar. Growth in the early instars was normal with 13 per cent sugar in the diet, but after the fourth instar this amount in most cases proved inadequate for complete development. Good growth and complete development occurred with 26 per cent. On a diet containing 39 per cent, some individuals developed completely but at a slower rate throughout.

2. The amounts of sugar/cellulose mixtures eaten during 24 hr by groups of hungry fifth-instar *Schistocerca* hoppers were studied with a view to assessing the effect on feeding of the phagostimulatory properties of sugar. Considerable amounts of pure cellulose were taken. Maximal intake occurred where sugar constituted 5 or 10 per cent of the mixture and only slightly less was taken where it was 2 per cent or 20 per cent. As it was also shown that third-instar hoppers at first grew well on a synthetic diet devoid of carbohydrate, it was concluded that the failure to grow on diets lacking carbohydrate was a result of strictly nutritional deficiency rather than a consequence of a failure to eat caused by unpalatability.

3. The effects on growth and development of different levels of sugar therefore showed that carbohydrate was essential for locusts, and were suggestive of an increased requirement during the later instars.

4. The utilizability of various carbohydrates and related substances was examined. The pentoses D-xylose, L-arabinose, D-ribose, and L-rhamnose were not available to either species. Both species utilized the hexoses glucose and fructose well, and D-mannose partially; *Locusta* utilized neither L-sorbose nor D-galactose, but while *Schistocerca* failed to utilize the former, it perhaps utilized galactose in low concentration. The disaccharides maltose, cellobiose, trehalose, sucrose, lactose, and melibiose, and the trisaccharides melezitose and raffinose were all utilized by both species, as were yellow and white dextrin, and potato and soluble starch. The availability of these complex carbohydrates indicates the possession by locusts of a wide array of digestive carbohydrases including the unusual β-glycosidases. The sugar alcohols sorbitol, mannitol, and meso-inositol were all utilized to some extent by both species, but whereas *Schistocerca* completed development with α-methyl-mannoside the growth of *Locusta* was, if anything, inhibited by this glycoside. The lipid substances, olive oil, palmitic,

and stearic acid were unable to replace carbohydrate, and glycerol was not utilized. Where carbohydrate was replaced by extra protein growth was, if anything, inhibited.

5. The ability of locusts to utilize carbohydrates is discussed in relation to previous studies on other insects. In their versatility locusts rank with stored products insects such as *Tenebrio* rather than the phytophagous species.

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STUDIES ON THE DERMESTID BEETLE *TROGODERMA GRANARIUM* EVERTS—IV. FEEDING, GROWTH, AND RESPIRATION WITH PARTICULAR REFERENCE TO DIAPAUSE LARVAE

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Abstract—Feeding, growth, respiration, and behaviour have been studied in the diapause and non-diapause larvae and in the post-larval stages of the Khapra beetle. In any one instar of a non-diapause larva, both feeding and respiration increase towards the middle of the instar and fall again towards the end. The relationship of the respiration curve to the type of food is discussed. It is apparent that the larva uses more oxygen when feeding and assimilating food than it does when re-organizing at ecdysis and metamorphosis. The diapause larva stays in a refuge for long periods in a dormant condition without feeding, with its rate of respiration reduced much below that of other post-oval stages. If the dormant larva is disturbed daily or denied access to a refuge, this rate is increased only slightly, even though the larva is capable of walking about. The dormant larva moults occasionally, although its rate of respiration increases little at the time of moulting. The function of the extra moults may be partly to facilitate changes in the size and behaviour of larvae. The diapause larva feeds for short periods between long periods of dormancy. Feeding is accompanied by a large increase in the rate of respiration, just as it is with a non-diapause larva. It appears that this feeding during diapause greatly extends the time a larva can live in diapause. When an experimental treatment is altered, with the result that the rate of respiration is changed, the change in rate is often delayed until after the larva has moulted. The extension of larval life by dormancy and slow respiration, with occasional short periods of rapid respiration associated with feeding, is regarded as a true facultative diapause. The endocrine control of the diapause and of the moulting during diapause is discussed.

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INTRODUCTION

Trogoderma granarium Everts, the Khapra beetle, is a pest of stored cereals and many other food materials. At favourable temperatures, some larvae pupate about a month after hatching (BURGES, 1957), whilst others leave the food when they are apparently fully grown and cluster for long periods in refuges such as the cracks and crannies in the fabric of the food stores (BURGES, 1959b). It seems likely that this delay in development is a facultative diapause. However, observation of groups of clustering larvae has shown that some larvae moult and leave their refuges to feed occasionally and that, if disturbed, all are active enough to search for new refuges (BURGES, 1959a). This behaviour poses the question of whether the larvae are in a true state of diapause and, more particularly, whether they enter the very inactive physiological condition typically associated with diapause. Accordingly the behaviour, feeding, growth, and rate of respiration of a small

number of larvae from clusters have been compared in detail with that of non-diapause larvae and other developmental stages.

For the present purposes, the term 'non-diapause' will be used for larvae which pupate promptly on reaching maturity without clustering; 'diapause larva' will refer to a larva with delayed pupation whether it is in the act of clustering or of feeding; 'dormant larva' will designate a clustering diapause larva.

METHODS AND APPARATUS

Insect material, food, and containers

In food stores dormant larvae aggregate in clusters, but the behaviour of individuals within a cluster is variable. At irregular intervals some of the larvae move away from the clusters to feed and then return (BURGES, 1959a). Thus some individuals in a cluster have not fed for several months, whereas others in the same cluster have fed recently. Probably their rates of respiration vary, so that daily measurements of the respiration of a whole cluster would be unsatisfactory. Since experiments showed that the behaviour of isolated diapause larvae appeared to cover the same range as that of larvae in clusters, it was decided to study isolated larvae.

The daily observations described below were made on two non-diapause larvae, bred singly in tubes from the hatching of the eggs, also on twelve diapause larvae taken from cultures 3 months after all the non-diapause insects had been removed as pupae. The insects were bred, and all the observations were carried out at 30°C and 70 per cent r.h.

The larvae were kept in two sorts of container. Eight of the diapause larvae were kept singly in 3×1 in. glass specimen tubes; the other four diapause larvae and the two non-diapause larvae were confined singly in $0{\cdot}5 \times 0{\cdot}5$ in. tubes, each in a respiration chamber of a respirometer. A layer of wheat, two grains deep, was used as food in the big tubes. In the small tubes, there was room for only two grains and the embryos of these were damaged with a knife to ensure that the larvae could attack them readily. The tubes were covered with cotton cambric to prevent the escape of larvae.

Refuges

Refuges were provided for the larvae, those used in the large tubes being termed 'large refuges' and those in the small ones 'small refuges'. A large refuge comprised two rectangular pieces of cardboard, $2 \times 5 \times 0{\cdot}16$ cm, lined with cotton cambric and held apart along one long edge by a narrow strip of the same cardboard, the whole being held together by elastic bands at each end. This refuge provided a narrow crevice of triangular cross-section, in which larvae of all sizes would remain (BURGES, 1959a). A small refuge was made by removing the lead from a section of wooden pencil, 0·6 cm long, and stopping up one end with a cellulose acetate cement. The section was then cut into halves longitudinally. The halves were held together with a very small elastic band, so that the refuge could be dismantled easily to remove the larva.

Daily observations

Each tube was examined daily to find whether the larva was in a refuge or on the food.

The growth of the four diapause and the two non-diapause larvae each kept separately in a respirometer was measured daily by weighing each larva to ±0·01 mg. After it had been weighed, a larva was returned to the position that it occupied before it was disturbed.

Each pair of grains from the small tubes in the respirometer were weighed daily to ±0·01 mg to give a direct measurement of feeding. Even though the wheat was kept at a controlled humidity of 70 per cent r.h. except when it was being weighed, its moisture content changed slightly from day to day, consequently the weight losses observed cannot be ascribed entirely to feeding. Increases in the moisture content at a time when the larva is not feeding provide an estimate of the error. These appear in Figs. 2, 4, and 5 as negative amounts of food eaten. Errors of 0·05 mg were quite frequent and the largest was 0·16 mg. Consequently a larva was not considered to have fed unless the observed weight loss of the grain was greater than 0·10 mg. A line has been drawn at this level on Figs. 2, 4, and 5.

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A larva produces large numbers of compact faecal pellets, which can be easily counted to provide an indirect and slightly delayed measure of the amount of feeding. Since only two wheat grains were used in each small tube, in the respirometer, all the pellets could be found easily and all the daily numbers are shown in Figs. 2, 4, and 5. The pellets were sieved from the wheat in each large tube and tipped on to a grooved perspex disc for counting. Preliminary tests showed that a few pellets stuck to the grain at sieving, but were removed at subsequent sievings. The maximum number of pellets left behind on the wheat after the first sieving was fifteen, therefore any number below this is not considered to be a true zero and is omitted from Fig. 3. Pellets were rarely found in either type of refuge.

The daily rates of respiration of the two non-diapause and of four of the diapause larvae were measured in a respirometer capable of recording up to 500 mm^3 of oxygen. Tests proved that the oxygen consumed by two wheat grains or by a large quantity of faecal pellets was negligible.

Respirometer

The respirometer is illustrated in Fig. 1. The design is based on a constant-pressure apparatus described by DIXON (1951). A manometer, containing odourless distillate coloured with sudan III, connects two chambers immersed in a water bath. One chamber contains the insect, two wheat grains, and a small refuge, all in a small tube, which rests on glass beads bearing a film of caustic potash to absorb carbon dioxide and to control the humidity at 70 per cent r.h. (SOLOMON, 1951). The other chamber contains duplicate articles but no insect. At the end of the respiration period, the volume of oxygen used by the insect is given by the change in level required in a graduated column of mercury to restore the oil-level in the manometer.

A number of special features are incorporated to obtain the necessary sensitivity. The volume of the respirometer is minimized. An extra arm is introduced outside the water bath (compensation tube in Fig. 1) to approximately equalize the volumes

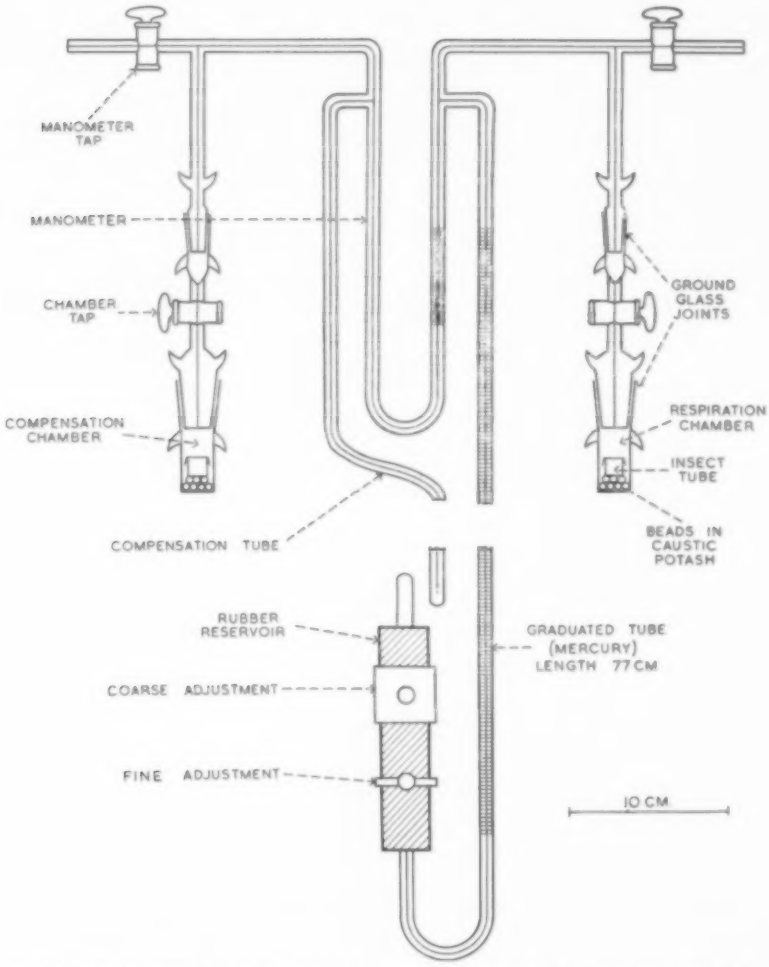

FIG. 1. Respirometer drawn to scale in one plane. In practice the chambers are behind the manometer and immersed in a water bath to a level just above the top joints.

of air at room temperature. The whole apparatus is kept in a constant-temperature room maintained at 30°C.

The volumes of oxygen are quoted for 30°C and 760 mm pressure.

To allow daily measurements to be made with several separate larvae in different chambers, an extra joint is incorporated above each chamber. This makes it possible

to connect and disconnect several pairs of chambers to the manometer one after another.

A series of sensitivity tests was conducted without insects. In eleven test blanks with a pair of chambers kept on the manometer overnight, a mean change of volume of 1·0 mm³ was observed. The largest was 2·2 mm³ which is regarded as the maximum error. Values as low as 3 mm³ were occasionally obtained later

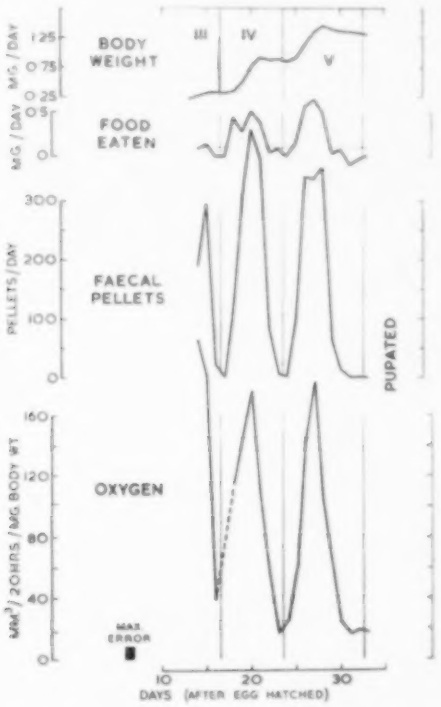

FIG. 2. Live weight, feeding, and respiration of a non-diapause male larva. Moults are shown by vertical lines and larval instars by III to V. A broken line indicates that one value is not available. The volume of oxygen is expressed at 30°C and 760 mm pressure.

with dormant larvae and the maximum error per milligram of larval weight is shown in Figs. 2, 4, and 5 by the thickness of a short line. Further tests showed that storing the chambers overnight on a rack in the water and subsequently connecting them to the manometer slightly increased the error. Therefore the chambers containing the dormant larvae, which were expected to give low values, were left on the manometer overnight. When the chambers were stored on the rack, the respirometer was slightly sensitive to changes of atmospheric pressure, because there were small differences in the volumes of the chambers. This error was eliminated by equalizing the volumes with glass beads.

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Since respiration was measured each day over a period of about 20 hr, the volumes of oxygen have been corrected to 20 hr. During this time, the greatest decrease (3 per cent) in the percentage of oxygen in a respiration chamber and the corresponding reduction in pressure (23 mm) were too small to influence the rate of respiration of the insect (KENNINGTON, 1953, 1957).

Full details of the development and use of the respirometer are given in a thesis (BURGES, 1956).

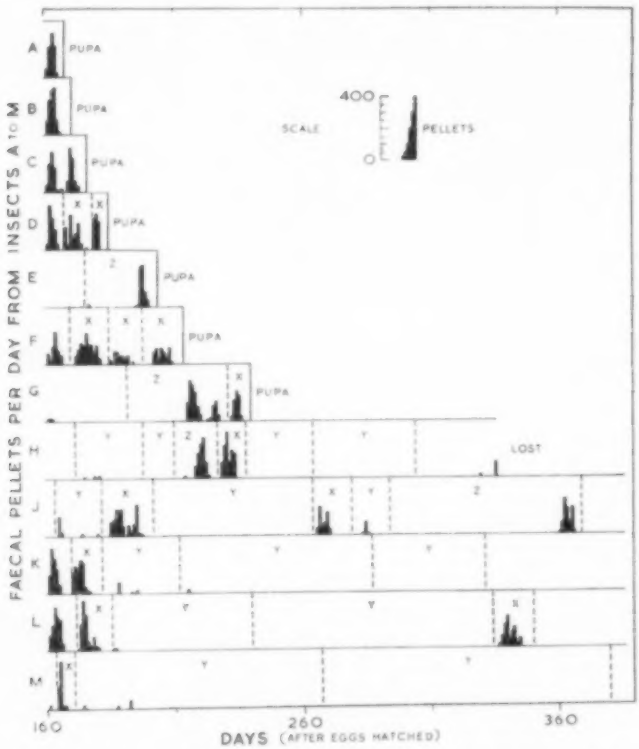

FIG. 3. Daily production of faecal pellets (black histograms) by twelve isolated diapause larvae, each with a refuge (larvae G and M were without refuges for short periods given in Fig. 4 and Table 3). Numbers of pellets below fifteen are not shown. Each moult is represented by a broken vertical line. Larvae E, G, H, and M were kept in the respirometer most of the time. The complete instars marked X, Y, and Z are classified as feeding, dormant, and mixed feeding and dormant respectively.

RESULTS

Non-diapause larvae

Very similar data were obtained from the two isolated non-diapause larvae, one male and the other female. Fig. 2 illustrates the results for the male from the third larval instar to pupation.

The general shapes of the two curves for the rate of feeding, estimated by the loss in weight of the wheat and by the production of faecal pellets, are very similar both to each other and to the curve of oxygen consumption. All three curves rise to a peak near the middle of each instar. Respiration falls to low values near each

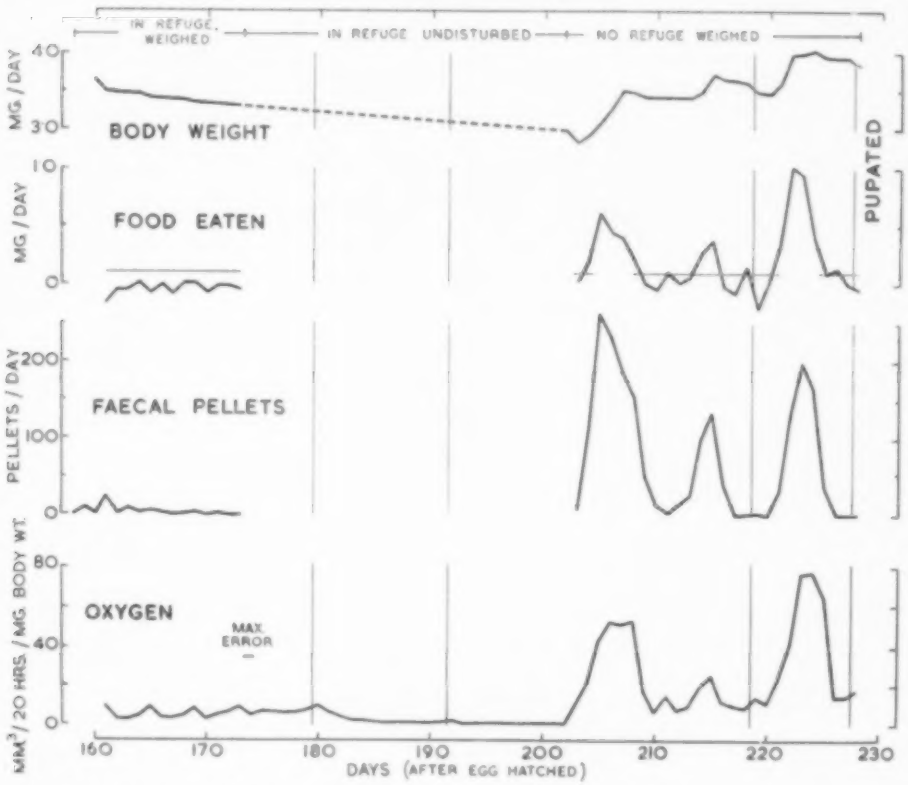

FIG. 4. Live weight, feeding, and respiration of larva G, a female diapause larva. The experimental treatments are shown along the top of the figure. The broken line indicates when the larva was not weighed. Moults are shown by vertical lines.

moult and no feeding takes place then. Obviously the rate of respiration depends very closely on the rate of feeding. While the larva is feeding actively, its body weight rises rapidly; in the absence of feeding, it falls slightly.

Diapause larvae

The numbers of faecal pellets produced per day by the twelve diapause larvae are shown in Fig. 3 and the feeding, respiration, and body weight of diapause larvae G and H, which were kept in the respirometer, are illustrated in Figs. 4 and 5.

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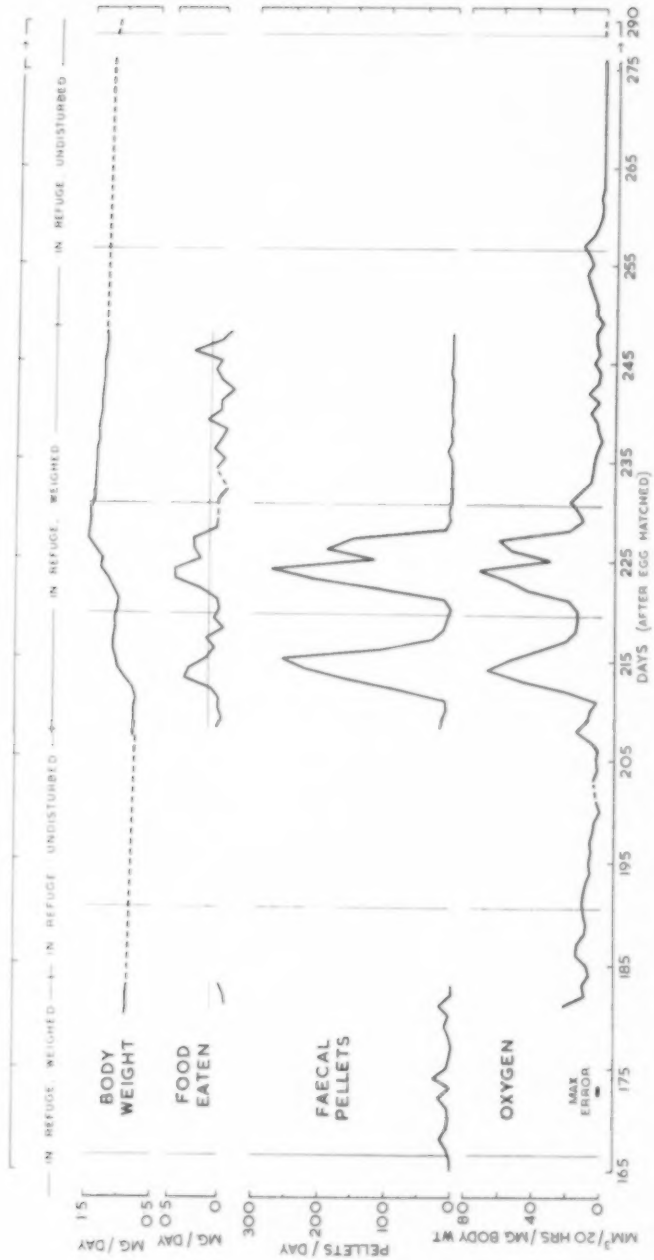

FIG. 5. Live weight, feeding, and respiration of larva H, a male diapause larva. The experimental treatments are shown along the top of the figure. Broken lines indicate the values are not available. Moults are shown by vertical lines.

As expected from earlier experiments (BURGES, 1959a), several larvae were soon induced to pupate by their sudden removal from the stock cultures and the sudden provision of new food after they had been in diapause in the cultures for over 3 months (Fig. 3). Before pupating, larvae A to C resumed feeding for a short period. Larvae D and F pupated after a longer feeding period interrupted by two or three moults. This feeding presumably replenished their food reserves, which must have been depleted during diapause. The other seven larvae also fed occasionally for one or more short periods. During feeding periods, the larvae were usually found among the wheat grains and their oxygen consumption and weight changed in the same way as in the non-diapause larvae (Figs. 2, 4, and 5). In addition, seven of the larvae spent one or more long periods of dormancy without feeding, during which they were usually found in the refuges, their body weights falling slowly and their rates of respiration being exceptionally low compared with those of non-diapause larvae.

Occasionally during the dormant periods, which often lasted over several instars, small numbers of faecal pellets were deposited, indicating that a little feeding took place (Fig. 3). Conversely, during a well-defined feeding period, a larva sometimes ceased to produce pellets for a few days in addition to the gaps in pellet production caused by moulting (Fig. 3); this certainly indicates a cessation of feeding because simultaneously the body weight stopped increasing, the weight of the grain ceased to drop, and the consumption of oxygen fell (e.g. days 209 to 213 in Fig. 4). These minor discrepancies do not appear to have any great significance.

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The twelve diapause larvae moulted at irregular intervals and their behaviour varied in different instars. Between them they had thirty-six moults into a further larval stage and seven into the pupal stage. Of the thirty-one complete larval instars marked X, Y, and Z in Fig. 3, thirteen short ones were spent feeding as in non-diapause larvae (X), fourteen of variable length were passed in the dormant condition with little or no feeding (Y), and four were spent dormant for a variable time then feeding for a short time (Z).

Thus extra instars occur in the diapause larva. Combined, these form long dormant periods interspersed between short feeding periods, so that the larval life is greatly prolonged in comparison with the non-diapause larva.

Effect of disturbing dormant diapause larvae

If dormant diapause larvae are left undisturbed in their refuges, they appear to move about very little. When they are placed on a balance pan for weighing, however, they often walk over the pan quite actively. This stimulated activity must increase their rate of respiration at the time and the increased respiration may continue while the animal is in the respirometer between weighings. Therefore the oxygen consumptions of larvae G, H, and M in small refuges over 'disturbed' periods with four or more consecutive daily weighings were compared with oxygen consumptions during similar 'undisturbed' periods without weighing or any sort of disturbance.

The results are given in Tables 1 and 2. Larva M consumed very little oxygen when undisturbed, but during subsequent periods of daily disturbance it consumed six times as much in one experiment and three times as much in another (Table 1).

TABLE 1—OXYGEN CONSUMED BY DORMANT LARVAE IN SMALL REFUGES WHEN A DISTURBED PERIOD FOLLOWED AN UNDISTURBED ONE

Larva	Disturbed	Consecutive daily periods of 20 hr	Mm^3 O_2/mg body wt./20 hr (Mean ± standard error)	P
M, expt. 1	No	22	1·14 ± 0·03	0·001
	Yes	14	6·50 ± 0·67	
M, expt. 2	No	19	1·38 ± 0·27	0·001
	Yes	12	3·94 ± 0·35	

These differences were both highly significant in separate analyses of variance for each experiment (following MATHER, 1949). In three experiments with larvae G and H, the order of the treatments was reversed, i.e. the period of daily disturbance was given first. After disturbance was stopped, the rates of respiration of the larvae eventually fell by more than a half (Table 2, $P = 0{\cdot}01$–$0{\cdot}001$). However,

TABLE 2—OXYGEN CONSUMED BY DORMANT LARVAE IN SMALL REFUGES WHEN A DISTURBED PERIOD PRECEDED AN UNDISTURBED ONE

Larva	Disturbed	Consecutive daily periods of 20 hr	Mm^3 O_2/mg body wt./20 hr (Mean ± standard error)
G	Yes	14	6·05 ± 0·44
	No, pre-moult	5	7·27 ± 0·77
	No, post-moult	21	1·71 ± 0·41
H, expt. 1	Yes	4	12·68 ± 1·55
	No, pre-moult	8	11·67 ± 1·23
	No, post-moult	13	5·02 ± 1·03
H, expt. 2	Yes	18	4·63 ± 0·63
	No, pre-moult	8	8·21 ± 1·01
	No, post-moult	8	2·12 ± 1·05

on all three occasions the falls were delayed until after the larvae had moulted, although in a later experiment the respiration of larva M fell without a delay and without a moult (Table 3, f and g).

Thus the disturbance of a dormant larva by weighing it once daily resulted in a greater average oxygen consumption during the rest of the day compared with days on which the larva was not disturbed, but the fall in the rate of respiration, caused by stopping daily weighing, was sometimes delayed until after a moult.

Even though a disturbed dormant larva consumed from two to six times as much oxygen as an undisturbed dormant larva, this increase was small in comparison to the increase caused by feeding (Table 4; Figs. 4 and 5).

TABLE 3—EFFECT OF A REFUGE ON THE OXYGEN CONSUMED BY DORMANT LARVA M

Treatment	Disturbed	Consecutive daily periods of 20 hr	Mm^3 O_2/mg body wt./20 hr (Mean ± standard error)	*P*
a. Refuge	No	19	1·38 ± 0·27	0·001
b. Refuge	Yes	12	3·94 ± 0·35	0·001
c. Tube	No, pre-moult	6	9·71 ± 0·51	0·001
d. Tube	No, post-moult	18	1·81 ± 0·31	0·5
e. Refuge	No	17	2·06 ± 0·34	0·01
f. Tube	No	5	3·60 ± 0·67	0·02
g. Refuge	No	7	1·09 ± 0·59	

TABLE 4—OXYGEN CONSUMED BY VARIOUS DEVELOPMENTAL STAGES OF *T. granarium*

Stage	No. of experiments	Consecutive daily periods of 20 hr	Mm^3 O_2/mg body wt./20 hr (Mean ± standard error)
Undisturbed dormant larva	3	42	2·9 ± 1·0
Disturbed dormant larva	3	36	7·8 ± 2·5
Undisturbed dormant larva after disturbance and before a moult	3	21	9·0 ± 1·3
Non-diapause larva near a moult	4	17	14·1 ± 0·7
Female pupa	1	5	18·2 ± 1·5
Male pupa	1	6	27·0 ± 1·7
Virgin adult	2	12	26·2 ± 2·0
Peak feeding of diapause larva	3	11	67·7 ± 3·8
Peak feeding of non-diapause larva	2	15	114·4 ± 11·8

Effect of a refuge on dormant diapause larvae

If diapause larvae are not given a refuge, they feed more, but they do not pupate sooner (BURGES, 1959a). To find whether a dormant diapause larva will respire slowly if it is not allowed access to a refuge, the oxygen consumption of larva M was measured while the larva was occupying a small refuge and while it was kept in a glass tube with neither refuge nor food.

The order of the treatments in the experiment is given in Table 3. The basal rate of respiration of the undisturbed, dormant larva in a refuge was measured for 19 days (Table 3 a), then the larva was disturbed daily to encourage it to feed if it wanted to. As expected, the rate of respiration increased after disturbance

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(Table 3 b), but the larva did not feed during the 12 days. Then the refuge and the food were removed, leaving only the larva in the tube (undisturbed). This caused a further increase in the oxygen used (Table 3, c), until the larva moulted, after which the respiration rate returned to near the basal level (Table 3, d). When the refuge was replaced, there was no marked change in the rate of respiration (Table 3, e). This suggests that, without a refuge, the respiration had reached a low basal level. In contrast, when the experiment was repeated with the omission of the period of disturbance, there was a small, but significant, increase in the respiration rate as soon as the refuge was removed and then a decrease without a moult when the refuge was replaced (Table 3, f and g).

Thus the rate of respiration of a dormant larva is low even without a refuge and may be as low as that observed when a refuge is present.

Effect of clustering and feeding on survival

The effect of the clustering behaviour and of feeding on the survival of diapause larvae was evaluated. Diapause larvae were allowed to aggregate in five large refuges placed in a large culture at 30°C and 70 per cent r.h. after all the non-diapause insects had been removed as they pupated. Three of these refuges were then placed each in a 2 lb jam jar containing fresh food. The fourth was placed in a jar without food. The fifth refuge was dismantled and the larvae emptied into another jar where they were kept with neither refuge nor food. Regular observation showed that of the 354 larvae given both food and a crevice, only a few died or pupated during the first year, fifty-five were still alive after 4 years and 150 had pupated. Of the sixty-six larvae given a crevice but no food, three were alive after 23 months and only one had pupated. All of the twenty-eight larvae given neither a crevice nor food died in 11 months. Obviously the occasional feeding protects the larvae against starvation. The evidence suggests that a diapause larva denied access to a refuge sometimes respires less slowly than a larva resting in a refuge (Table 3, f and g) so that presumably the free larva uses up its food reserves more quickly and therefore succumbs to starvation sooner.

DISCUSSION

Nature of the diapause

Diapause can be defined as an arrest of growth and development, which persists in environments favourable for normal growth and development until the animal is subjected to certain well-defined conditions. These conditions permit some physiological change, which enables growth to be resumed as soon as the environment favours it. In a review of the physiology of diapause, Lees (1955) states 'The arrest of growth (and the decline in locomotory and feeding activity which accompanies the onset of diapause in larval and adult insects) is invariably associated with a striking fall in the level of metabolism'.

During the life-cycle of *T. granarium*, the rate of respiration fluctuates violently. This suggests great changes in the rate of metabolism. Table 4 shows that respiration is much less during undisturbed larval dormancy than during any

other post-oval condition. The undisturbed dormant larva spends long periods without feeding, moving, or changing morphologically, therefore its very low rate of respiration is probably at a basal level, sufficient only to maintain life. The mean level of respiration during the pupal period is six to nine times as high (Table 4). In comparison, freshly pupated pupae of the Cecropia silkworm consume

TABLE 5—OXYGEN CONSUMED BY DIAPAUSE STAGES OF INSECTS

Mm^3 O_2/mg body wt./ 20 hr	Temp. (°C)	Stage	Species	Reference
0·15	18*	Pupa	Butterfly, *Endromis versicolora* (L.)	AGRELL (1951)
0·21	18	Pupa	Butterfly, *Phalera bucephala* (L.)	AGRELL (1947–8)
0·30	24	Pupa	Giant silkworm, *Platysamia cecropia* L.	SUSSMAN (1952)
0·33	25	Pupa		SCHNEIDERMAN and WILLIAMS (1953)
0·34	25	Egg	Grasshopper, *Melanoplus differentialis* Thomas	ROBBIE (1941)
0·5–0·6	15	Pupa	Moth, *Daseochaeta alpium* Osbek	KOZHANTSHIKOV (1938)
0·7–0·9	15	Egg	Gypsy moth, *Lymantria dispar* (L.)	KOZHANTSHIKOV (1938)
0·9–1·2	15	Pupa	Moth, *Colocasia coryli* (L.) (quoted as *Calocasia*)	KOZHANTSHIKOV (1938)
1·0–1·2	15	Prepupa	Sawfly, *Croesus septentrionalis* (L.)	KOZHANTSHIKOV (1938)
0·8–1·4	15	Pupa	Moth, *Loxostege sticticalis* (L.)	KOZHANTSHIKOV (1938)
1·0–1·6	15	Pupa	Moth, *Apatele rumicis* (L.) (quoted as *Acronycta*)	KOZHANTSHIKOV (1938)
1·36	25	Egg	Silkworm, *Bombyx mori* L.	CHINO (1958)
1·7	22	Pupa	Moth, *Celerio euphorbiae* L. (quoted as *Deilephila*)	HELLER (1926)
1·90	18	Larva	Beetle, *Byrrhus pilula* L.	AGRELL (1947–8)
2·8	20*	Adult	Tenebrionid beetle, *Opatrum sabulosum* (L.)	ÊDEL'MAN (1951)
2·9	30	Larva	Khapra beetle, *Trogoderma granarium* Everts	BURGES (present paper)
3·3	—	Larva in cocoon	Larch sawfly, *Pristiphora erichsonii* (Htg.)	MCDONALD and BROWN (1952)
3·4	20*	Adult	Tenebrionid beetle, *Anatolica eremita* Stev.	ÊDEL'MAN (1951)
7·7	27	Adult	Sweetclover weevil, *Sitona cylindricollis* (Fahr.)	DAVEY (1956)

* Personal communications.

on average nine times the oxygen used by diapause pupae (SCHNEIDERMAN and WILLIAMS, 1953). Furthermore, the rate of respiration of the diapause *T. granarium* larva is similar to that of diapause stages of three species listed in Table 5, much less than one, but much greater than thirteen. There are large differences in the temperatures at which the rates of respiration of the species listed in Table 5 were measured. Although temperature influences the rate of respiration of diapause

insects much less than that of active insects, it is likely to have some effect on diapause insects. For instance, the average oxygen consumption of the Mud-dauber wasp *Sceliphron caementarium* (Dru.) at 35°C is one and a half times that at 28°C and six times that at 25°C (BODINE and EVANS, 1932). The oxygen consumption of *T. granarium* was measured at a higher temperature than that given for any other species included in Table 5, but it must be remembered that *T. granarium* has a high range of temperature (21–40°C). It can be concluded that the low rate of metabolism of the dormant *T. granarium* larva, persisting in temperatures, humidities, and foods favourable for rapid growth, is strong evidence that the dormancy is a true diapause.

The reduced metabolism of a dormant larva of *T. granarium* is accompanied by a distinct behaviour pattern. The larva leaves its food and moves into its hiding place or refuge, where it stays for a long time. In cultures, larvae cluster in refuges, each larva orientated to allow as much as possible of its body surface to touch something. This appears to be a powerful thigmotaxis accompanied by gregariousness. Most species of insect in diapause reduce their feeding and eventually their locomotory activity. This reduction is sometimes accompanied by gregariousness as in some ladybirds and in the Monarch butterfly, *Danaus plexippus* L. (WILLIAMS, 1958).

Occasionally a diapause larva of *T. granarium* leaves a cluster, feeds for a short period, then joins a cluster again. This is comparable to the feeding of diapause larvae of the moth, *Dendrolimus pini* L. (GEÏSPITZ, 1949), and of the Dragon fly, *Anax imperator* Leach (CORBET, 1956), suggesting that larvae of *T. granarium* enter a similar state of diapause to larvae of these species. However, the diapause of *T. granarium* is obviously less well defined than that of species which neither feed nor moult during their period of diapause, e.g. the Warehouse moth, *Ephestia elutella* Hübner (WALOFF, 1949).

When diapause larvae of *T. granarium* are starved, most are prevented from pupating and their survival times are greatly reduced compared with those of diapause larvae allowed to feed. All of the seven diapause larvae that pupated, while under individual observation in the presence of food, fed before doing so (Fig. 3). Thus the feeding periods are certainly of functional value in prolonging survival and probably also in enabling larvae to pupate after partial depletion of the food reserves during dormant diapause.

The basal level of respiration during the diapause of different species is a highly variable feature (Table 5). LEES (1955) considers this level to be an indication of the rate of mobilization of the insect's food reserves, a feature controlling the duration of survival of the diapause insect, which itself is a reflexion of the intensity of diapause. When starved at 30°C, some *T. granarium* larvae in a refuge survived for 23 months. Some species probably cannot survive in diapause as long as these larvae. For example, during about 7 months in diapause, the beetles *Anatolica eremita* Stev. and the female of the mosquito *Culex pipiens* L. use two-thirds or more of their stored fat, therefore they probably cannot survive much longer than this (ÉDEL'MAN, 1951; BUXTON, 1935). These are probably in a shallower

state of diapause than *T. granarium*. Many species can survive much longer periods in diapause: LEES (1955) listed eight species able to last for over 2 years. The diapause stages of these species are probably less mobile and in a deeper state of diapause than larvae of *T. granarium*.

It can be concluded that larvae of *T. granarium* undergo a definite facultative diapause, with a distinct associated behaviour pattern: the diapause is better defined than in some species, but less well defined than in others.

The cycle of feeding and moulting

During the different developmental stages of both diapause and non-diapause *T. granarium*, the rate of respiration provides a measure of the level of metabolism associated with the various body activities. The low maintenance level of the diapause larva in a refuge is on average 2·9 mm^3 per 20 hr per mg of body weight (Table 4). During the pupal period and near the moulting time of non-diapause larvae, the respiration rate is five to nine times higher than the basal level: presumably this higher level is associated with re-organization. During the larval feeding periods, peak values of twenty-three to thirty-nine times the maintenance level were obtained. The dominant activities at this time are presumably the chewing and intake of food (wheat), and its assimilation.

HOWE (1948) obtained very similar results for the carbon dioxide production of a much larger beetle, *Tenebrio molitor* L., which also feeds on cereal products. BIRCH (1947) measured the oxygen consumption of the weevil *Calandra oryzae* L. and the Bostrichid *Rhyzopertha dominica* F., both of which feed on cereal grains. Unfortunately the ratios of larvae to prepupae in his groups of insects were not known exactly, but his results are not inconsistent with the occurrence of maximum respiration during peak feeding, just before the pre-pupa is formed.

In contrast, ZWICKY and WIGGLESWORTH (1956) obtained very different results with larvae of the bug *Rhodnius prolixus* Stål. During an instar, the rate of respiration is lowest near the middle and highest at moulting. In each instar, the bug acquires its blood meal very quickly and soon concentrates it into a small quantity of protein-rich food for storage in the alimentary canal. No more activity is required for feeding, and the act of feeding is separated in time from digestion. This allows assimilation and re-organization to take place simultaneously, forming a peak of respiration at moulting time. In the middle of the instar and before the next blood meal, the rate of respiration is very low, coinciding with an apparent absence of anabolism, morphogenic change, and feeding. The intake of food during an instar of the four beetles mentioned above is too great for extensive storage in the gut during ecdysis, therefore feeding and assimilation must be largely coincident with each other, but mostly separated in time from ecdysis. Thus the relationship between the rate of respiration and the cycle of feeding and moulting appears to depend to some extent on the type of food.

HEIST (1957) found that the oxygen consumption of the blowfly *Lucilia sericata* (Meigen) (quoted as *Phaenicia*) was smaller during the middle feeding period of larval life than during the periods of wandering at the beginning and end.

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However, the measurements were made with larvae briefly deprived of a feeding medium, so that the values during the feeding period may be artificial. Nevertheless, the results suggest that active wandering may cause a higher rate of respiration than the feeding of the blowfly larva.

Howe (1948) obtained a slight increase in the respiration of the large beetle, *T. molitor*, at ecdysis, probably caused by the physical effort of shedding the skin. Such an increase with *T. granarium* would be too small to be detected with the respirometer used.

Function of moulting during diapause

A moult can be regarded as a time of change in the life-cycle of an insect, most noticeable at the metamorphosis of the Holometabola, but still noticeable in their larval stages and in the larvae of the Heterometabola. The ecdysis of *Rhodnius* and of the non-diapause *T. granarium* larva allows the insect to increase in volume at convenient intervals. Similarly a moult during a dormant period of a diapause *T. granarium* larva probably allows it to decrease in size as it consumes its food reserves, although the morphological change at this time is probably small, since it is accompanied by only a very small increase in respiration. A number of other changes related to the behaviour of the insects are associated with the ecdysis in the diapause *T. granarium* larva. For example, all five larvae, which fed and then entered a dormant period (one doing so twice and another three times, Fig. 3), moulted very soon after feeding ceased, before becoming dormant; dormant larvae frequently moulted just before beginning a feeding period (Fig. 3); finally, in dormant larvae, subjected to daily disturbance followed by a period without disturbance, the decrease in their oxygen consumption was usually delayed until after moulting (Table 4).

The diapause larvae of many species of insects do not undergo extra moults, even though intermittent feeding may take place. These mostly belong to families in which most species studied have a fixed or limited number of moults. The Dermestids, however, commonly have a very variable number of moults so that changes in the behaviour of larvae of *T. granarium* are more likely to be associated with moulting than in families with a more constant number of moults.

Endocrine control of moulting and diapause

The diapause of some species of insect is broken by the same endocrine system as that controlling moulting, e.g. the diapause of pupae of the Cecropia silkworm is broken by the secretion of the brain hormone, which stimulates the prothoracic glands to release a hormone, which in turn initiates the pupa-to-adult moult (Williams, 1947). In the diapause larva of *T. granarium*, however, larva-to-larva moults take place without breaking diapause (Fig. 4). In some species, while the moulting hormone from the brain causes the insect to moult, the amount of juvenile hormone present and its timing determine the degree of differentiation toward the adult condition taking place at the moult (Wigglesworth, 1954). Furthermore, Wigglesworth (1958) and Schneiderman and Gilbert (1958) have shown that the topical application of extracts containing juvenile hormone cause patches of

cuticle to bear juvenile characters at the next moult. Thus it appears that larva-to-larva moults in the diapause *T. granarium* are likely to be caused by the moulting hormone in the presence of active juvenile hormone. The intervals between the moults of diapause larvae are usually much greater than those of non-diapause larvae, indicating that the release of the moulting hormone is considerably delayed during diapause. Probably the diapause is broken by the release of the moulting hormone together with a decrease in the activity of the juvenile hormone. In some larvae pupation takes place at the first moult following the application of the extrinsic cause of breaking the diapause; in others one or more larva-to-larva moults intervene before pupation (Fig. 3), which suggests that the rate or the time of loss of juvenile hormone activity varies.

YOSHIO WAKU, of the Kyoto Technical University, Kyoto, Japan, has informed me in private correspondence that he has evidence of endocrine activity in diapause larvae of the moth *Plodia interpunctella* Hübner. His histological observations of larvae in diapause revealed an increase in the activity of the corpus allatum, which suggests that this organ plays an important role in the introduction and maintenance of the diapause. A difference in the rate of respiration between the larvae destined to enter diapause, and those not, can be seen well before the observed onset of diapause. The diapause of this insect has many similarities to that of *T. granarium*, but the larvae neither moult nor feed during diapause.

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TYROSINASE IN THE SILKWORM DURING THE PUPATION PERIOD

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Abstract—The change in tyrosinase activity in silkworm blood from the full-grown larval stage to pupation follows a V-shaped curve. In the integument the curve is almost reversed, and the activity is lowest just after pupation. Tyrosinase activity in the blood plasma gives an almost similar curve to that of whole blood, whereas in the haemocytes the activity is rather constant and the ratio of the activity in blood plasma to whole blood was very low at pupation compared with any other time. Tyrosinase could be observed only in oenocytoids (and/or phagocytes of the pupal type); the other types of haemocytes scarcely stained with Nadi reagent or catechol solution. During pupation the darkly stained haemocytes amount to several per cent of all haemocytes. The change of tyrosinase activity in acetone powder of the blood is fairly different from that of intact blood, and the amount of copper found in blood plasma is higher than in the whole blood during pupation.

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INTRODUCTION

DURING recent years there has been much interest in the nature and the process of hardening and darkening of insect cuticle, which takes place after moulting or pupation. The important role of tyrosinase in darkening and hardening has been noted by many investigators (RICHARDS, 1951; KAWASE, 1955, 1956).

DENNELL (1947) discovered a kind of large oval haemocyte (termed 'oenocytoid') in the larva of *Sarcophaga*, giving a deep blue colour with the Nadi reagent. According to him, they secrete tyrosinase into the blood and then disappeared before pupation, whereas, in the larva of *S. bullata*, 'spherule cells' are supposed to contain tyrosinase (JONES, 1956).

In the silkworm, *Bombyx mori*, the tyrosinase activity of the blood during the pupation period has been studied by HARIZUKA (1947) and ITO (1953, 1954), but the tyrosinase activity of the different blood components has not been examined. For further understanding of pigment formation in the cuticle, it seems desirable to acquire more information concerning the physiological details of connexions between tyrosinase and blood components. The aim of the present paper is to investigate the fluctuation of the tyrosinase activity in the blood, haemocytes, and integument, and its relation to pupal pigment formation.

MATERIALS AND METHODS

N122 × C115 and some other strains of silkworms were used as materials. Measurements of tyrosinase activity were made by the oxygen uptake of enzyme preparations using a Warburg respirometer. The main compartment of the

manometric flask contained 1 ml of M/10 phosphate buffer (pH 6·8) and 0·5 ml of enzyme preparation. The centre well contained 0·3 ml of 20 per cent KOH, and 0·5 ml of a 0·3 per cent catechol solution was placed in the side arm. The flasks were shaken at about 100 oscillations per minute at 37°C. Enzyme preparations are as follows: (1) Integuments of eight larvae or pupae at the same stage were cleaned, homogenized with nine parts in weight of distilled water, and centrifuged for 5 min at about 3000 rev/min to use the resulting supernatant as the enzyme solution. (2) To protect blood from melanization, it was collected in a cold glass tube. Both blood and blood plasma, the latter being obtained by centrifuging the blood for 10 min at 3000 rev/min to remove haemocytes, were diluted with 9 vol of distilled water to make the enzyme preparations of blood and blood plasma. As it was not possible to separate haemocytes only from blood, the difference in the enzyme activity between blood and blood plasma in equal volume was shown as the tyrosinase activity of the haemocytes. (3) An aqueous solution of acetone powder obtained from 1 ml of blood plasma and whole blood dissolved in 10 ml distilled water was also used as the enzyme solution. As tyrosinase in *Bombyx mori* increases in activity slightly on being left (ITO, 1953; SAKAGUCHI, 1958), the procedure described above must be done at low temperatures as quickly as possible.

Haemocytes in natural blood and blood fixed with methanol were tested with the Nadi reagent or a catechol solution (DENNELL, 1947; JONES, 1956). The copper content in blood and blood plasma was tested by a colorimetric method using diethyldithiocarbamate (SANDELL, 1950).

RESULTS

Tyrosinase activity in the blood and integument

Tyrosinase activity in the blood and integument was measured from the mature larval stage to the pupal (Fig. 1). Fig. 1 indicates that the tyrosinase activity in the blood decreases gradually until the late spinning period, then increases abruptly and reaches the highest level just after pupation, resulting a V-shaped curve. In integument the activity becomes high at the early spinning stage and thereafter decreases, reaching the lowest level just after pupation.

Tyrosinase activity in the blood plasma

Strictly speaking, the difference in tyrosinase activity between whole blood and blood plasma in equal volume is not attributable to the tyrosinase activity of the haemocytes. In this experiment, however, the difference was ascribed to the enzyme activity of the haemocytes, because it was not possible to isolate haemocytes from the blood.

As shown in Fig. 2, tyrosinase activity in the blood plasma at the pupation period follows a curve similar to that of the whole blood, but at a lower level. Besides, it is clear that at the stage of low tyrosinase activity, the activity in the blood plasma is almost negligible, whereas the activity of haemocyte tyrosinase is rather constant.

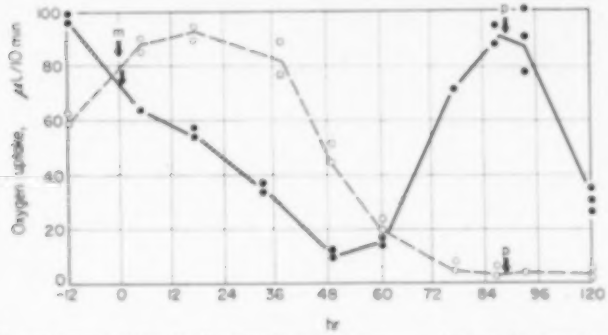

FIG. 1. Tyrosinase activity in the blood and integument during the pupation period. ●, blood; ○, integument; m, mounting; p, pupation.

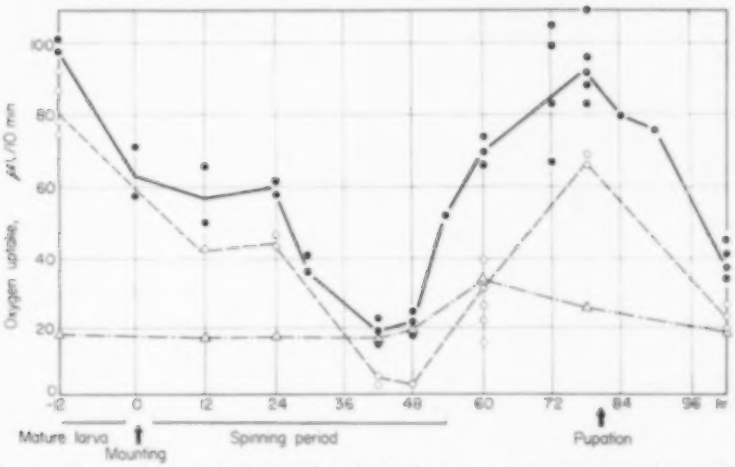

FIG. 2. Tyrosinase activity in blood and blood plasma during the pupation period. ●, whole blood; ○, blood plasma; △, whole blood; – blood plasma.

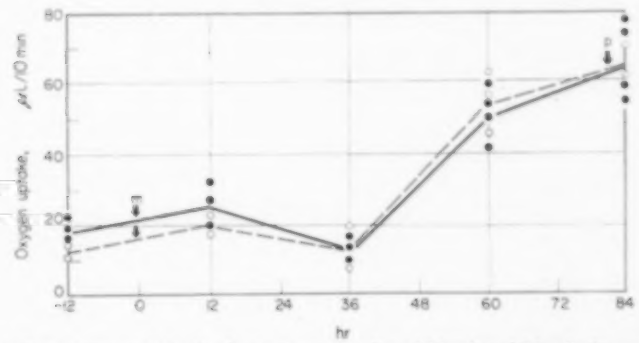

FIG. 3. Tyrosinase activity in the acetone powder of blood and blood plasma during the pupation period. ●, whole blood; ○, blood plasma; m, mounting; p, pupation.

The tyrosinase activity of blood plasma and whole blood was also measured from the fourth larval ecdysis to the pupal stage (Table 1). It will be seen from Table 1, that the ratio of the activity of blood plasma to whole blood is very low at the late spinning period.

TABLE 1—COMPARISON OF THE TYROSINASE ACTIVITY OF BLOOD AND BLOOD PLASMA

Stage	Whole blood (μl/10 min)	Blood plasma (μl/10 min)	Blood plasma / Whole blood
Fourth moult (middle stage)	18·2	9·5	0·53
Fifth day, fifth instar	66·3	49·6	0·75
Spinning period (late stage)	26·2	4·9	0·19
Just after pupation	92·4	67·3	0·73
First day pupa	31·8	16·2	0·51

Note: Tyrosinase activity was indicated by oxygen consumption.

The tyrosinase activity in the acetone powder of whole blood and blood plasma is shown in Fig. 3. The change of both tyrosinase activities is fairly different from intact blood and blood plasma, especially at the mature larval stage.

Cytochemical observation of tyrosinase in the haemocytes

By cytochemical techniques, tyrosinase could be found in a large oval haemocyte (diameter: 10–15 μ) in the blood irrespective of the methanol treatment. It appears to be an oenocytoid (and/or a phagocyte of the pupal type). The other types of haemocytes such as proleucocytes, spherule cells, and plasmatocytes of NITTONO (1956) were scarcely stained with Nadi reagent or catechol solution (Fig. 4). Although the Nadi reaction takes place in the presence of cytochrome oxidase and tyrosinase (DENNELL, 1947), a positive Nadi reaction in haemocytes pre-treated with methanol, and also the darkening by catechol solution, indicate the existence of tyrosinase because cytochrome oxidase has been destroyed by the alcohol treatment. The number of darkly stained haemocytes comprises several per cent of all haemocytes, and the percentage is comparatively constant throughout the above-mentioned stage, unlike *Sarcophaga* (DENNELL, 1947; JONES, 1956).

Copper content in blood and blood plasma

It has been reported that silkworm and mammalian tyrosinase is a copper–protein compound, and the copper found in the blood is mainly that of tyrosinase (IWANARI, 1955). Inhibition or activation of tyrosinase in silkworm blood has been suggested by several writers (ITO, 1953; SAKAGUCHI, 1958). In order to determine whether or not the above-mentioned fluctuation of tyrosinase activity is due to the fluctuation of tyrosinase itself or some other factors such as glucose dehydrogenase activity (DENNELL, 1949), the copper content in blood and blood

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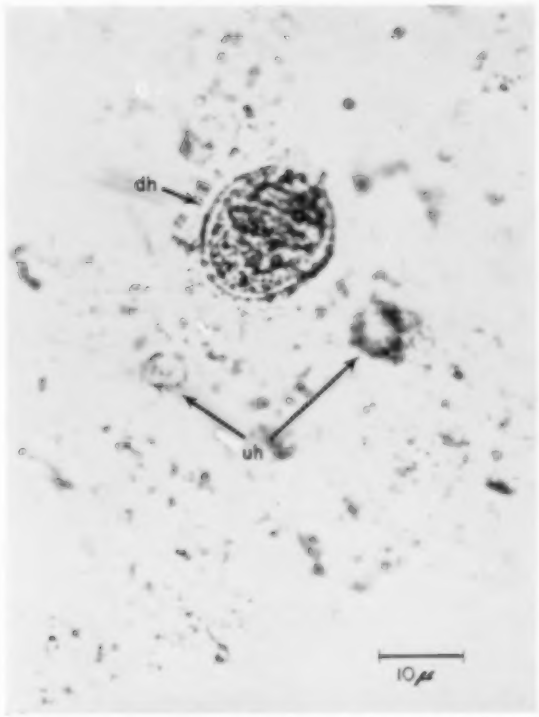

FIG. 4. The haemocytes immersed in catechol solution. dh, darkened haemocyte; uh, unstained haemocyte.

plasma was tested during the pupation period. As indicated in Fig. 5, copper increases gradually throughout the stage, and the copper content in blood plasma is higher than that in whole blood.

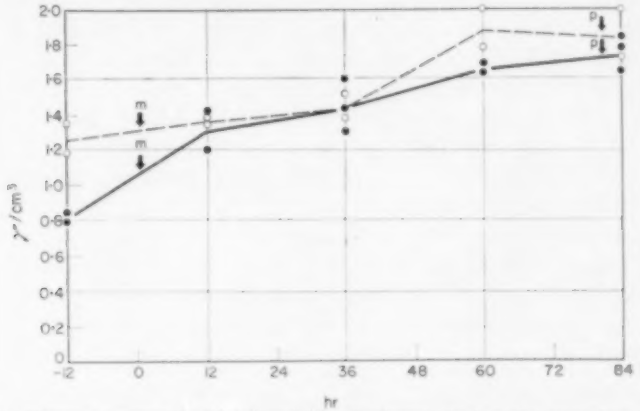

FIG. 5. Copper content in blood and blood plasma during the pupation period. ●, whole blood; ○, blood plasma; m, mounting; p, pupation.

DISCUSSION

It has been shown that tyrosinase plays an important part in melanin formation in the silkworm cuticle (KAWASE, 1955, 1956). However, the precise mechanism of the darkening of the cuticle remains uncertain at present.

As shown in Fig. 1, it is of interest that the fluctuation of the tyrosinase activity of blood and integument is almost reversed at pupation. From this it cannot be necessarily inferred that the interchange of tyrosinase between integument and blood occurs. ITO (1954) reported that the fluctuation of tyrosinase activity in the acetone powder prepared from silkworm blood is little compared with that in the blood itself. From the difference in activity between fresh blood and acetone powder, he suggested the presence of a factor masking or inhibiting the tyrosinase in the blood, especially at pupation. However, the present experiments with acetone powder gave a very different result, i.e. tyrosinase activity in the acetone powder prepared from blood plasma and whole blood is very low compared with that of intact blood and blood plasma from the full-grown larval stage to the late spinning period, although at the prepupal stage the difference in activity between them is not clear. Furthermore, the fluctuation is fairly similar to that of the copper content. These results suggest the possibility that the tyrosinase of the mature larval stage is considerably different from that of the later period.

It has been reported that the copper content in the blood plasma is higher than that of blood during the fifth instar (IWANARI, 1956). During the pupation period also, as already noted, the copper content in the blood plasma is higher than that of the blood, but the tyrosinase activity in the blood plasma is less than that of the blood during the same period. In other words, the copper content in the haemocytes is very low as compared to that of the blood plasma. For a further

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understanding of this difference, several points may be considered, namely, whether the tyrosinase in the haemocytes has little copper, or the tyrosinase activity in the blood plasma is inhibited or masked by the other factors. If the cytochrome oxidase of the silkworm blood contains copper, as shown by WAINIO *et al.* (1958) in the ox heart, the phenomenon may be explained partly by the copper content of cytochrome oxidase found in the blood plasma.

In the silkworm only oenocytoids (and/or phagocytes of the pupal type) have tyrosinase as in *Sarcophaga*, in which tyrosinase originates in the blood oenocytoids (DENNELL, 1947). However, the appearance and disappearance of the haemocytes containing tyrosinase during the pupation period could not be observed in the silkworm, unlike *Sarcophaga* (DENNELL, 1947). Therefore, it may be considered that in the silkworm the origin of tyrosinase in the blood remains unclear, although some part of the tyrosinase may be derived from that of the haemocytes.

As mentioned above, the relationship between tyrosinase and copper in the haemocytes and blood plasma during the pupation period are so confused that detailed experiments on these subjects are required.

Acknowledgements—The author wishes to express his most cordial thanks to Prof. K. HASEGAWA for his valuable suggestions, and to Dr. Y. NITTONO, Sericultural Experiment Station, Shinjo, for his valuable advice on the classification of haemocytes.

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PHYSIOLOGY OF SAWFLY METAMORPHOSIS—I CONTINUOUS RESPIRATION IN DIAPAUSING PREPUPAE AND PUPAE

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Abstract—The respiration of diapausing prepupal stages of the sawfly *Cephalcia abietis* L. and pupae of *Dolerus nigratus* F. has been studied by means of a volumetric microrespirometer with a special adaptation for concurrent measuring of respiratory quotients. Contrary to findings on most diapausing pupae, a continuous respiration without pronounced CO_2 bursts has been found. For comparison, the respiration of lepidopterous pupae of *Bupalus piniarius* L. and *Hyphantria cunea* Drury of the same body size as the sawflies was investigated. In these a distinctly discontinuous respiration has been found, the amount of CO_2 released in the inter-burst period being, however, greater than in large lepidopterous pupae.

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INTRODUCTION

SPECIFIC aspects of the gaseous exchange of insects in the diapause period of low metabolism have been studied by different authors. Using the diaferometric method, PUNT (1944, 1950, 1956) found respiration to be cyclic in certain resting insects. He described a periodic release of a great quantity of carbon dioxide during short intervals ('bursts') alternating with long intervals of very slow output, and associated with a corresponding rhythm in the opening of the spiracular valves. Such discontinuous CO_2 release has been found in several diapausing insects since that time. The oxygen consumption of these insects is nearly continuous according to SCHNEIDERMAN and WILLIAMS (1955) and BUCK and KEISTER (1955) —(investigated manometrically) or shows just a small increase in the burst period according to PUNT, PARSER, and KUCHLEIN (1957)—(investigated diaferometrically). In this way a much greater amount of oxygen enters the body during the inter-burst period than the amount of CO_2 released, hence the respiratory quotients appear to be very low (cf. AGRELL, 1951) in discordance with the actual relations in insect metabolism. To obtain the correct values of respiratory quotients the whole cycle has to be taken into account (SCHNEIDERMAN and WILLIAMS, 1955).

Typical discontinuous respiration may be seen in large lepidopterous pupae. However, it appears also in other stages as in diapausing larvae and in adults (PUNT, 1950). Most authors have supposed that discontinuous respiration is widespread in resting insects. However, due to technical difficulties involved in measuring very low metabolic rates, so far only species of large size have been studied. PUNT (1950) attempted the diaferometrical determination of CO_2 output

in diapausing eonymphs of the sawfly *Lophyrus pini* L., but he found the specimens too small to permit reliable measurements.

Using volumetric microrespirometers of high sensitivity the author has tried to solve the question of whether discontinuous respiration occurs also in such a group as sawflies diapausing mostly in the prepupal stage in soil under conditions of great humidity and relatively low oxygen concentration. As controls, small lepidopterous pupae of about the same size as the sawflies were also studied.

MATERIALS AND METHODS

The experimental specimens were kept in round pits in plaster blocks regularly moistened, at about 20°C. In the present investigation two species, of different diapause habit, were used. The first, *Cephalcia abietis* L., diapauses as two prepupal stages, eonymph and pronymph, and the diapause usually lasts 2 years. These stages are usually in the 150 mg wt. range. The other, *Dolerus gonager* F., spends diapause as an overwintering pupa of 50–60 mg wt. In addition, the respiration of small lepidopterous pupae, *Bupalus piniarius* L. and *Hyphantria cunea* Drury, in winter diapause, has been studied.

Gaseous exchange was determined volumetrically using a modification of the DRASTICH (1924) microrespirometer. The vessels had a total volume of from 1 to 3 ml, depending on the respiratory rate of the animal being studied. A drop of kerosene stained with Sudan III served as an index. The readings were performed upon the mercury in the measuring capillary with a sensitivity of 0·05 mm^3/hr. The whole device was constructed of thin glass capillaries, the index capillary being attached to the respiratory vessels with thin plastic tubing. The whole thing was submerged in a water bath with temperature regulation in the range of $\pm 0{\cdot}1$°C.

For concurrent determinations of respiratory quotients and CO_2 output the following adaptation was used (Fig. 1). In the bottom of the respiratory vessel there is a special centre well (a) with upper rim polished. The centre well may be tightly closed with a circular glass cover (b) cut from a cover slip and provided with two little U-shaped iron hooks (c). By means of a magnet (m) the centre well may be closed or opened by the glass cover without opening the respirometer vessel or changing its volume. The glass cover is moistened on the lower side with thin paraffin oil so as to ensure a good seal. The time necessary for complete absorption of CO_2 in a given vessel is determined initially; for the vessels used in the present experiments 5 min periods for absorption were found satisfactory. The centre well was filled with 10% KOH. A paper moistened with 5·5% H_2SO_4 is placed in the upper part of the respiratory vessel. These concentrations have the same tension of water vapour (BUCK and KEISTER, 1955). The animal to be analysed is placed in the middle of the vessel upon a strip of thin plastic.

The measurements may start with closed centre well (CO_2–O_2). Then O_2 consumption alone is obtained upon opening the centre well by means of the magnet so that CO_2 absorption results. In the experiments repeated 5 min readings of CO_2–O_2 were made, and after this absorption of CO_2 also during 5 min was followed. Thus O_2 consumption was obtained for the whole period including

all readings of CO_2–O_2. The CO_2 release rate was then calculated from the CO_2–O_2 and the O_2 values. Such alternating CO_2 and O_2 determinations may continue as long as the O_2 concentration in the vessel has not changed considerably. The device makes possible the measurement of very small gaseous exchanges as in diapausing insects, in insect eggs, and isolated insect organs where it is impossible to use the pumping systems described by KRÜGER (1935) and JANDA (1957).

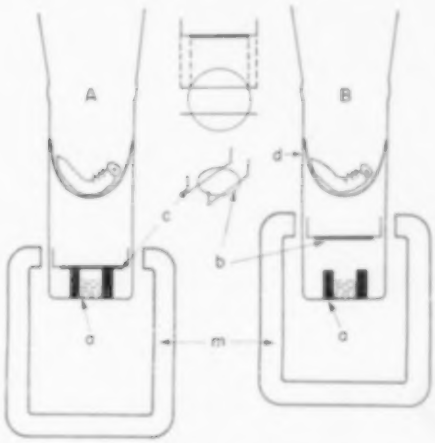

FIG. 1. Respiratory vessels of volumetric microrespirometer and the adaptation for measuring respiratory quotients. A = Respiratory vessel with closed centre well; B = Same with opened centre well; a = centre well with ground upper rim; b = glass cover; c = small iron pieces cemented to the glass cover; d = strip of plastic; m = magnet. Explanation in text.

Further, it makes it possible to determine changes in respiratory quotients within rather short intervals. It permits measuring both CO_2 output and O_2 consumption on the same individual without interrupting the experiment, and it prevents the accumulation of CO_2 in the respiratory vessel. The electromagnetic mechanism is easily adapted to various kinds of volumetric and manometric apparatus.

RESULTS

Most of the experiments were made with the sawfly *Cephalcia abietis* L. This species diapauses the first year as an eonymph and the second year as a pronymph, as do certain other sawflies (SLÁMA, 1958). The eonymph is still mobile, whereas the pronymph is nearly motionless and thus is more convenient for respiration experiments. The metabolic rate is greater in eonymphs than in pronymphs. The rate of oxygen consumption is 120–130 $mm^3/g/hr$ in eonymphs and 60–70 mm^3 in pronymphs at 20°C (respectively 60 $mm^3/g/hr$ and 30–40 mm^3 at 10°C). Oxygen consumption is very stable in pronymphs and more variable in eonymphs, in connexion with their spontaneous activity. No significant discontinuity was observed in oxygen consumption, and carbon dioxide is released

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continuously also (Fig. 2). During experiments lasting several hours with twenty eonymphs and twenty pronymphs of *Cephalcia abietis* L. no bursting registered either at 20°C or 10°C. Hence, in contrast to insects with discontinuous respiration,

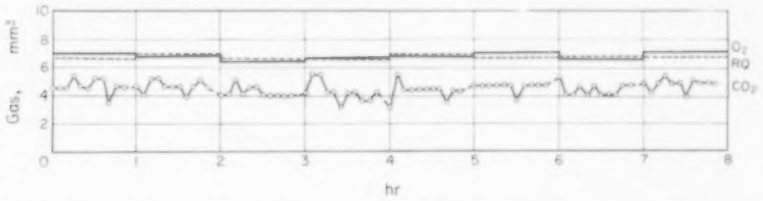

FIG. 2. The O_2 consumption, CO_2 output, and the respiratory quotients of diapausing pronymphs of the sawfly *Cephalcia abietis* L. Values indicate mm³ of gas per hour per specimen. (Weight 144 mg.)

where a very low respiratory quotient during interburst alternates with a high respiratory quotient during the burst, the prepupal stages of *Cephalcia* have a constant respiratory quotient (ca. 0·68).

As mentioned above, typical discontinuous respiration has been found so far mostly in diapausing pupae. PUNT (1950) found cyclic CO_2 release also in hibernating caterpillars and adult butterflies, but showing many more bursts than in hibernating pupae. Hence it appeared interesting to know if the continuous

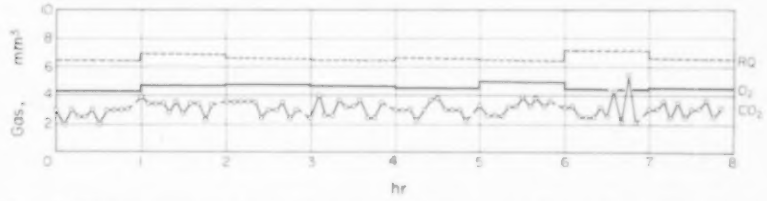

FIG. 3. The O_2 consumption, CO_2 output, and respiratory quotients of diapausing pupae of the sawfly *Dolerus gonager* F. Values indicate mm³ of gas volume per hour per specimen. (Weight 67 mg.)

respiration in the diapausing sawfly *Cephalcia* is a specific feature of the prepupal stages only, as these resemble larvae more than pupae. Therefore, further experiments with diapausing pupae of the sawfly *Dolerus gonager* F. were made during its winter diapause. The total metabolism of these pupae in the given period is approximately the same as that of pronymphs of *Cephalcia*. The oxygen consumption is 50–60 mm³/g/hr at 20°C and 30–40 mm³ at 10°C. As Fig. 3 shows, both the O_2 consumption and CO_2 release are continuous here too.

Since the size of the investigated diapause sawflies with continuous CO_2 release is much less than that of most insects where discontinuous CO_2 release has been found (e.g. the pupae of *Dolerus* are about 100 times smaller than those of the cecropia silkworm), it could be supposed that continuous CO_2 release in diapausing sawflies is associated with small size. This possibility was tested with diapausing

pupae of *Bupalus piniarius* L. weighing about 50 mg and on *Hyphantria cunea* Drury of about 100 mg. As shown in Fig. 4, CO_2 release in these small lepidopterous pupae is distinctly discontinuous, even though the rates of CO_2 release

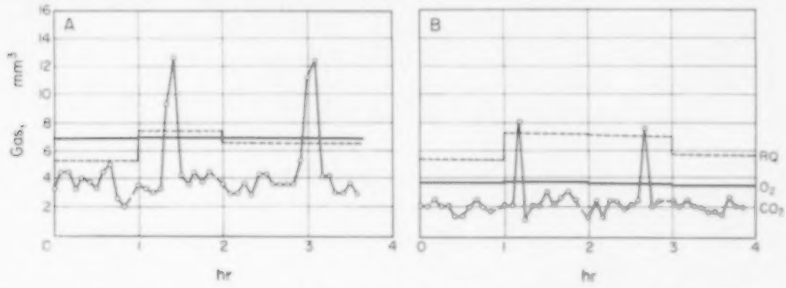

FIG. 4. The O_2 consumption, CO_2 output, and the respiratory quotients in diapausing pupae of *Hyphantria cunea* Drury (A) and *Bupalus piniarius* L. (B). Values indicate mm^3 of gas volume per hour per specimen. The specimen of *Hyphantria* weighed 113 mg, that of *Bupalus* 57 mg.

during burst and interburst periods are not as different as in some larger Lepidoptera. Incidentally, the records show that the respirometer is fully capable of detecting discontinuous respiration in small insects.

DISCUSSION

Since discontinuous respiration has been supposed to be a widespread phenomenon in resting insects, it is somewhat surprising to find continuous respiration in such group as the sawflies, which possess a true diapause. It seems worthwhile, therefore, to consider sawfly respiration and ecology in relation to four factors which have been suggested in the literature as possibly being correlated with cyclic CO_2 release.

The first of these factors is water balance. BUCK and KEISTER (1955) and BUCK (1957, 1958b) have suggested that discontinuous respiration is an adaptation for minimizing the water loss that is usually so serious a problem in diapausing insects. Rather different relations confront sawflies diapausing in the soil 10–20 cm below the surface under conditions of high humidity. In the sawfly *Cephus cinctus* Nort., for example, it has been found that the diapausing prepupal stage not only does not lose water but actually is able to absorb water from the environment (CHURCH, 1955). Likewise, under laboratory conditions diapausing sawfly stages must be kept in a high humidity, and preliminary experiments show that their resistance against the water loss is distinctly smaller than in the cyclically respiring lepidopterous pupae. These facts therefore fully support the idea that discontinuous respiration is concerned with minimizing water loss.

A second possible factor in the non-occurrence of discontinuous respiration in sawflies might be the ambient oxygen tension. According to SCHNEIDERMAN and WILLIAMS (1955) increase in pO_2 favours the periodical release of CO_2 and, on the other hand, a lowering of O_2 causes an increase in rate of CO_2 release during the

interburst period and may finally change cyclical respiration into a more or less continuous type. Hence the low oxygen tension in the underground cells where sawflies diapause might also be involved in the observed continuous respiration.

Thirdly, some peculiarities in metabolism are to be mentioned which distinguish diapausing sawflies from other diapausing insects. In diapausing pupae of the cecropia silkworm with typical discontinuous respiration, inactivation of the cytochrome c–cytochrome oxidase system has been found by SCHNEIDERMAN and WILLIAMS (1954). The pupae are very insensitive to inhibitors of cytochrome oxidase. On the contrary, diapausing prepupal stages of the sawfly *Pristiphora erichsonii* Htg. have been found to be no more sensitive to cyanide than the non-diapausing stages (MCDONALD and BROWN, 1952).

The fourth factor influencing respiration, not only in sawflies but probably in all small insects, seems to be the rate of metabolism in relation to the body size. For example, PUNT (1950) found that an increase in metabolism causes increase in burst frequency, decrease in burst size, and increase in relative rate of CO_2 release during the interburst period. Similarly, SCHNEIDERMAN and WILLIAMS (1955) found that increasing the metabolic intensity in cecropia pupae by raising the temperature, terminating diapause, or mechanical injury, tended to induce continuous respiration. Since it is well known that intensity of metabolism in small insects is relatively higher than in larger ones, and since diapausing prepupal stages of sawflies have approximately three to five times the rate of gaseous exchange per unit weight than pupae of cecropia, it might be supposed that there is a connexion between the continuous type of respiration in diapausing sawflies and their relatively high metabolism. However, the measurements reported above on small lepidopterous pupae of *Bupalus* and *Hyphantria* show that small body size *per se* does not preclude the occurrence of cyclic CO_2 release in a diapausing insect, though the cycles (and the interburst R.Q.'s) may not be as exaggerated as in large pupae.

BUCK and KEISTER (1958) suggested that cyclical respiration might be evoked in insects with continuous CO_2 release by increasing the ambient pO_2 or by lowering of metabolism, and they succeeded in inducing discontinuous respiration in *Bombyx mori* (a normally non-cyclic species) by lowering the temperature. Although sawfly pupa fail to show cyclic respiration even at 10°C, this temperature is within the range of their normal environment, so that the possibility of discontinuous respiration in diapausing sawflies is not quite excluded. Possibly the answer may reside in the suggestion of BUCK and KEISTER (1958) and BUCK (1958a) that a condition for cyclical respiration is not low respiratory metabolism in itself but a low metabolic rate in relation to the O_2 supply.

SUMMARY

By means of volumetric microrespirometers the respiration of diapausing prepupal stages and pupae of sawflies *Cephalcia abietis* L. and *Dolerus gonager* F. was investigated. A description of a simple adaptation for a measuring respiratory quotient at short intervals is given.

Both oxygen consumption and carbon dioxide release in diapausing prepupal stages of *Cephalcia abietis* L. and in diapausing pupae of *Dolerus gonager* F. have been found to be continuous at 10–20°C, respiratory quotients being themselves steady around the value 0·68. Possible reasons for the occurrence of continuous respiration rather than the discontinuous respiration common in diapausing insects, are discussed from the point of view of water loss, ambient oxygen concentration, and rate of metabolism in relation to the body size.

In the small diapausing lepidopterous pupae of *Bupalus piniarius* L. and *Hyphantria cunea* Drury, used as controls, a cyclical respiration has been found. The rate of carbon dioxide release during the interburst periods is considerably higher, relative to the rate of O_2 uptake, than in large lepidopterous pupae, and the interburst respiratory quotient is likewise higher (mean value 0·5 as compared with 0·1 or even lower in the cecropia silkworm).

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